A Study on the Feeding of the Pot-bellied Seahorse (*Hippocampus abdominalis*): Reducing the Reliance on Brine Shrimp (*Artemia*).

By

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DECLARATION

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June, 2006.

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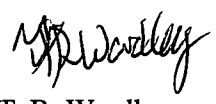

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ABSTRACT

The primary aim of this study is to reduce the reliance on *Artemia* during the culture of pot-bellied seahorses. The results demonstrate that reliance can be reduced significantly if not totally replaced. Early juvenile seahorses can be fed on alternative live diets such as copepods as the growth ($F = 0.054$, df 1, $p > 0.05$) and condition ($F = 0.416$, df 1, $p > 0.05$) of 3-week old seahorses fed copepods was similar to those fed enriched *Artemia.* It was also found that seahorses as young as newborns readily consumed copepods and gammarid amphipods (*Hippomedon* sp and *Biribus* sp) from the biofouling panels. Later juveniles can continue to be fed on biofouling crustaceans as the growth ($F = 0.982$, df 1, $p > 0.05$) and condition ($F = 7.401$, df 1, $p < 0.05$) of 17-week old seahorses fed biofouling was similar to those fed *Artemia* or weaned onto frozen diets.

The best predictor for determining prey size was the total length of the seahorse. Based on Cheeson's standardised forage ratio the preferred prey type of 5, 21 and 49 day old seahorses showed a particular preference for copepods with 21 and 49 day old seahorses also positively selecting both amphipod species (*Hippomedon* sp and *Biribus* sp). Larger 147 and 175-day old seahorses positively selected both amphipod species and 203-day old seahorses' positively selected *Biribus* sp and caprellids (*Caprella* sp).

Thirteen week old seahorses were weaned onto either a frozen mysid or amphipod diet with a nil, 10 day and 16 day weaning period and it was found that the growth of seahorses weaned onto frozen diets over a 16 day period had similar growth ($F = 83.922$, df 7, $p < 0.05$) to those seahorses fed enriched *Artemia.* It is possible that younger seahorses could be weaned onto frozen diets if appropriate sized feeds can be attained. It was also found that although a range of commercial enrichment diets had no affect on the growth ($F = 0.671$, df 5, $p > 0.05$) and condition ($F = 1.637$, df 5, $p > 0.05$) of seahorses, *Artemia* should be enriched as the liver condition in seahorses fed unenriched *Artemia* was poor and the optimal feed rate for seahorses was between 5 and 7 % body weight day^{-1} ($F = 0.47$, df 5, $p < 0.05$).

This study also examined the anatomy of the digestive system and ontogenetic development of digestive enzymes to provide a better understanding of the pot-bellied seahorses' nutritional requirements and digestive capacity. Seahorses are released with a near fully developed digestive tract and could be said to be fully developed between day 21 and 35 after the intestinal valve develops on day 7 and the intestine starts to loop around itself on day 21. Trypsin, lipase and amylase were present at every stage studied. The presence of enzymes in unfed newborn seahorses indicates that they are capable of digesting protein, lipid and carbohydrates prior to the onset of feeding, however the digestive system may not be fully functional until around day 28 to 35 when enzyme activities appeared to plateau. It was also found that trypsin and lipase activities were greater than amylase activities indicating that seahorses rely more heavily on protein and lipid than carbohydrate for their early nutrition.

Lastly a cost benefit analysis of alternative feed sources was prepared which demonstrated that significant savings in costs can be achieved.

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CHAPTER ONE

GENERAL INTRODUCTION

Seahorses (Syngnathidae) are a relatively new aquaculture species in a number of countries worldwide. Culture of these unique fish developed as a potential method of supplying product to markets while relieving pressure on wild stocks caused by high demand in the Traditional Chinese Medicine market (TCM) and aquarium trade. A number of small and large scale operations have been established over the last decade with the common objective being to determine whether seahorse culture is feasible and if so whether it is economically viable. Due to the significant number of papers that have been published world-wide on various aspects of seahorse husbandry since 1998, farming some seahorse species is possible but it remains expensive due to prohibitive feed costs. If seahorse culture is to become economically viable cheaper alternative diets are required.

Up until 1996 there was little data available on the exploitation of seahorses, the international aquarium trade and the Traditional Chinese Medicine market. A report based on limited customs statistics available at the time estimated that some 25 million seahorses were being harvested from the wild annually (National Geographic News, 2002). The need to conserve these unique animals was recognized and seahorses were placed on the IUCN (International Union for Conservation of Nature) red list as vulnerable.

Project Seahorse was also founded in 1996 and is an international organization committed to conservation and sustainable use of the world's marine ecosystems. Its seahorse program included research into the biology and ecology of seahorses, the co-ordination of seahorse aquarium husbandry, the monitoring of seahorse fisheries to regulate the harvesting of wild stocks to sustainable levels and encourages community-based conservation in fishing villages. Project Seahorse is also working with the TCM community in Hong Kong and plays a major part in policy development (www.projectseahorse.com).

A Marine Medicinals Conservation Program was launched by Project Seahorse in 1998 in partnership with WWF Hong Kong and Traffic East Asia. As a result of this program it was established that until more specific management tools are defined a minimum catch size of ten centimeters would assist the protection of seahorse populations by ensuring that seahorses are able to reproduce at least once before being harvested. This restriction was primarily directed at the seahorses species used in the TCM (*H. barbouri*, *H. comes*, *H. erectus*, *H. ingens*, *H. reidi* and *H. spinosissimus*). The Hong Kong and Chinese Medicines Merchants Association now promotes the ten centimeter minimum size limit (Whaley, 2004).

Interest in conservation of seahorses was heightened in November 2002 when Syngnathids were listed in Appendix II on the CITES list. Trade restrictions took effect on the 15th of May 2004 and countries now have to demonstrate that the export of seahorses will not be detrimental to the survival of seahorses in the wild ("non-detriment finding"). Environment Australia lists the conservation status of seahorses as Data deficient and populations were placed under the Australian Wildlife Protection Act in 1998 then under the Environment Protection and Biodiversity Conservation Act (EPBC Act) in 2001 (www.deh.org.au). A permit issued under section 14 of the Living Marine Resources Management Act 1995 is required to keep seahorses in Australia (www.dpiwe.tas.gov.au) and to import or export seahorses a permit issued under the EPBC Act 1999 is required (www.deh.org.au).

There are 10 species of seahorses in high demand for the medicinal market and include the Barbour's seahorse (*Hippocampus barbouri*), Reunion seahorse (*H. borboniensis*), Giraffe seahorse (*H. camelopardalis*), Tiger tail seahorse (*H. comes*), Lined seahorse (*H. erectus*), Sea pony (*H.fuscus*), Thorny seahorse (*H.*

histrix), Pacific seahorse (*H. ingens*), Kellogg's seahorse (*H. kellogi*), spotted seahorse (*H. kuda*), and the three-spotted seahorse (*H. trimaculatus*).

There are 20 species used in the aquarium trade and include the pot-bellied seahorse (*H. abdominalis*), Barbour's seahorse (*Hippocampus barbouri*), Reunion seahorse (*H. borboniensis*), short-headed seahorse (*H. breviceps*), Giraffe seahorse (*H. camelopardalis*), Knysa seahorse (*H. capensis*), Tiger tail seahorse (*H. comes*), Lined seahorse (*H. erectus*), Sea pony (*H.fuscus*), long-snouted seahorse (*H. guttulatus*), short-snouted seahorse (*H. hippocampus*), Pacific seahorse (*H. ingens*), Kellogg's seahorse (*H. kellogi*), spotted seahorse (*H. kuda*), slender seahorse (*H. reidi*), hedgehog seahorse (*H. spinosissimus*), West Australian seahorse (*H. subelongatus*), White's seahorse (*H. whitei*) and dwarf seahorse (*H. zosterae*).

Commercial farming is necessary to meet the high market demand for seahorses and reduce the unsustainable level of harvesting wild stock (Job et al., 2002; Warland, 2002). The concept of farming seahorses for trade has met with resistance from conservation groups, however many strategies need to be evaluated and implemented to protect these species.

A number of seahorse operations have commenced in the last 10 years and a number of research institutes have focused their work on the husbandry of seahorses. As stated earlier, the aim of these facilities is to determine whether seahorse aquaculture is technically feasible and, if so, whether it can be made economically viable. The commercial farming of seahorses, together with encouraging the world to purchase "captive bred seahorses" would be a step in the right direction towards conserving the over-exploited stocks of wild

seahorses. Captive bred fish which are already conditioned to aquarium environments are easier to maintain and feed than wild counterparts.

Facilities include Seahorse Aquaculture Pty. Ltd. (Beauty Point, Tasmania), now Seahorse Australia Pty. Ltd., which was the first commercial farm in Australia and was founded in 1998. It opened as a research and development facility to explore the feasibility of breeding seahorses in captivity and is currently focused on breeding, culturing and supplying superior disease free seahorses for the home aquaria (www.seahorseaquaculture.com.au). In the first years of operation Seahorse Australia produced about 400,000 seahorses a year. Other seahorse farms and research stations include South Australia Seahorse Marine Services (Port Lincoln, South Australia) which commenced farming seahorses in 1998 (www.saseahorse.com), Seahorse Ireland Ltd. (www.amdareef.com), The Seahorse Farm (Hawkes Bay, New Zealand) which opened in 2002 and is a division of HBA Aquaculture Ltd.

Seahorses are also produced at the Tropical Marine Centre (Hertfordshire, UK), Sceanic Aquarium (Ontario, Canada), Ocean Rider (Kailua-Kona, Hawaii) (www.oceanrider.com), a number of facilities in Queensland, China, Vietnam and the United States and at the National Institute of Water and Atmospheric Research (Matunga Bay, New Zealand) which has been developing the potential to culture seahorses since 1997. Seahorse World Pty. Ltd. (Beauty Point, Tasmania) opened in 2000 as a tourist venture and is dedicated to educating the world about the preservation and conservation of seahorses. Although Seahorse World Pty. Ltd. is not a production facility it still produces 10,000 to 20, 000 seahorses a year.

It has been said that "attempts to keep and/or culture seahorses generally ends in biological and/or economic failure" (Wilson & Vincent, 1998). However, due to the advancement of aquaculture and the continuation of studies in several countries including Australia, New Zealand, England, Malaysia, Singapore, Philippines, Vietnam, South Africa, China and United States (Forteath, 1997) the culture of a number of seahorse species (*H. abdominalis*, *H. barbouri*, *H. breviceps*, *H. capensis*, *H. comes*, *H. erectus*, *H. kuda*, *H. subelongatus*, *H. whitei*) has become possible.

The ability to successfully rear a number of seahorse species through multiple capture generations with high survival rates and good growth has come about by studies focusing on seahorse biology which has allowed tank densities to be determined and provided an understanding of the fishes' behaviour (Blake, 1976; Lovett, 1969; Perante et al., 2002). Studies on culture techniques and husbandry have led to the development of systems for newborns and grow out facilities and allowed for the optimal environmental parameters such as light, temperature, pH, and dissolved oxygen to be determined (Garrat, 1997; Lawrence, 1998; Lockyear et al., 1997; Payne, 2001; Sobolewski, 1997; Wilson & Vincent, 1998; Woods, 2000a; Woods, 2001).

Investigation into breeding behaviour and brood stock maintenance have made captive breeding a possibility (Boyden, 1995; Kvarnemo et al., 2000; Lawrence, 1998; Lockyear et al., 1997; Masonjones, 2001; Masonjones & Lewis, 1996; Sobolewski, 1997; Teixeira & Musick, 2001; Vincent et al., 1992; Vincent, 1994ab; Wilson et al., 2001; Woods, 2000ab). Studies on feed management and feed activity patterns have identified and tested feeds that promote good growth in different aged seahorses and have also provided knowledge on daily rations and feeding times (Job et al., 2002; Junyi et al., 2002; Payne, 2001; Ping-sun et al., 2002; Prein, 1995; Thompson, 1999). Other research on the growth of seahorses (Filleul, 1996; Shapawi, 2000), and their diseases (Vincent & Clifton-

Hadley, 1989) have refined husbandry and hygiene practices ensuring the welfare of seahorses under culture conditions.

The pot-bellied or big-bellied seahorse, *Hippocampus abdominalis* (Leeson, 1927) is a member of the family Syngnathidae and is one of the largest seahorse species. Although seahorses have not been taxonomically well defined there are reportedly 32 species world-wide of which 25 are found in Australian waters (Kuiter, 2000). Pot-bellied seahorses, which are the focus of this study, are distributed in waters around south-eastern Australia and New Zealand (Forteath, 1997; Lourie et al., 1999). They are large, smooth skinned species which range from pale, near white in colour to mottled yellow to variable brown with dark spots on their head and trunk and dark bands on their tail and have been reported to reach up to 35 cm in length (Francis, 1996). Pot-bellied seahorses are found at a depth of less than 50 m on bare sand, seagrass beds and around artificial structures (Forteath, 2000); they are exclusively carnivores feeding on a variety of small crustaceans (Filluel, 1996; Forteath, 1997) and are capable of multiple births in a season (spring to summer) with a gestation period of 32 days and produce between 400 to 600 offspring, which are 16 mm in length, each batch (Forteath, 1997; Kuiter, 2000).

At the University of Tasmania, Launceston pot-bellied seahorses have been studied for the last thirteen years (Forteath, 1997). During this period there have been 18 student degree projects dedicated to gathering knowledge on the culture of pot-bellied seahorses. Research has included aspects of reproductive ethology (Boyden, 1995), optimising growth (Filluel, 1996), diel feeding and activity patterns (Thomson, 1999), respiration and feeding (Boomsma, 2000), genetic and morphological variation (Armstrong, 2001), effect of dietary pigments on colour and adaptation to background (Wardley, 2001), respiratory physiology (Adams, 2002), swim bladder inflation, live transport (Lopez, 2002), (Florent, 2003), and predicting diet success (Wilson, 2003).

The University of Tasmania works collaboratively with Seahorse Australia Pty. Ltd. and Seahorse World Pty. Ltd. to refine the culture of pot-bellied seahorses and to date culture is technically feasible but remains uneconomical due to prohibitive feed costs. With this in mind the University and Seahorse World Pty. Ltd. put together a proposal for a Doctorate project with the main objective of finding and testing alternative diets for seahorses to reduce the reliance on *Artemia*. An APA (I) grant funded by both ARC (Australian Research Council) and Seahorse World Pty. Ltd. was awarded to the University in 2001. Since this project started Chris Woods from NIWA (New Zealand) has published a number of papers on alternative diets for pot-bellied seahorses.

The majority of cultured marine fish larvae are reared initially on a range of live feeds, which include copepods, algae, rotifers and *Artemia*. The successive use of live feeds depends on the size, age and development stage of the larvae. *Artemia* is the main live feed used in the culture of many larvae and early juveniles (Narcisso et al., 1999; Petkam & Moodie, 2001) because they are purchased as cysts which can be stored for years, cultured in large numbers and easily hatched (Payne & Rippingale, 2000; Stottrup, 2000).

Another favoured attribute of *Artemia* is that their quality or nutritional profile can be controlled through enrichment practices (enrichment emulsions, yeast, microalgae and fish byproducts) as *Artemia* are non-selective particulate feeders (Cho et al., 2001; Narcisso et al., 1999; Sorgeloos & Leger, 1992; Watanabe et al., 1980). The cost of *Artemia* cysts, however, can be prohibitive and expensive equipment and intensive labour is also required in culture, especially for grow-out to adults (Borlongan et al., 2000; Leu & Liou, 1992; Petkam & Moodie, 2001; Stottrup, 2000). The high market demand, uncertainty of supply (ie unreliable production in the wild) and prohibitive cost of culture make it essential to find economically viable alternative food sources to replace or reduce the reliance on *Artemia* in fish culture.

Extensive studies on diet and feeding behaviour have enabled many species of cultivated early stage marine fish to be weaned near metamorphosis onto cheaper, reliable artificial diets. Cultured pot-bellied seahorses are reliant on live feeds. Soon after release from the male's pouch early juvenile pot-bellied seahorses are able to feed on instar II *Artemia* and do not require the more complex feeding regime (including algae and rotifers) fed to other marine fish. This is possible because of their large size and advanced development on release from the pouch relative to many other marine fish species at hatch.

At Seahorse World Pty. Ltd. seahorses are fed instar II *Artemia* until they are four to six months old and then weaned onto adult *Artemia*. When this project started in 2001 both Seahorse Australia Pty. Ltd. and Seahorse World Pty. Ltd. were feeding late juveniles frozen mysids and supplementing the adults diet with live amphipods. There were no structured research projects on the effect of feeding frozen mysids and amphipods to seahorses at that time. This project was implemented to experimentally assess the effectiveness of such practices and to further develop the use of alternative diets. If seahorse culture is to be economically viable reliable alternative live diets, which could also be presented as frozen diets, are needed to totally or at least partially replace the need to culture *Artemia*. Ultimately, an artificial diet tailored to meet the requirements of cultured seahorses needs to be formulated.

The ability to formulate diets that fish will accept is facilitated by determining the feeding habits and behaviour of fish, their food preferences, feed intake, relationship between predator and prey size, the development of the digestive tract, ontogeny of gut enzymes, their nutritional profiles and behaviour of their prey (Baskerville-Bridges & Kling, 2000ab; Borlongan et al., 2000; Boulhic & Gabaudan, 1992; Clark et al., 1986; Coutteau et al., 1996; Hjelmand et al., 1993; Kjorsvik et al., 1991; Lauff & Hofer, 1984; Liang et al., 1998; Madrid et al., 1997; Miyazaki et al., 2000; Munilla-Moran & Stark, 1989; Planas & Cunha,

1999; Segner et al., 1993; Sanchez-Vazques et al., 1996; Swenson & McCray, 1996; Teshima et al., 2000; Walford & Lam, 1993). Some of these approaches will also be used in this project to assess the potential of evaluating artificial diets in the future and to better understand how seahorses select and utilize their diets.

Overall, the primary aim of this study is to reduce the reliance on *Artemia* during the culture of pot-bellied seahorses. Firstly trials will be conducted on pot-bellied seahorses fed different *Artemia* enrichment diets to assess growth, condition and survival rates. This experiment will be conducted because enrichment types vary and current data on growth curves and survival rates for seahorses are not based on acceptable scientific protocols. The most effective enrichment diet will then be used as a control or reference diet with which to compare the performance (growth and survival of seahorses) of other live and frozen diets. A range of daily *Artemia* rations will also be studied to ascertain optimal feed management; preferred prey type of seahorses will be assessed by using a selective index; the relationship between predator and prey size will be assessed to ensure appropriate size feeds are fed to seahorses of different age groups. During the frozen diet trial, weaning methods will be assessed to gauge the time a seahorse requires to accept a new feed and a workable weaning protocol will be determined. This project will also describe the mouth structure, anatomy of the digestive tract and development of gut enzymes over time so as to provide a better understanding of the feeding habits, nutritional requirements and digestive capacity of the pot-bellied seahorse (Bisbal & Bengston, 1995; Dinis et al., 1999). The latter aspects of the study will also provide information on when the gut is fully functional, which is important in the development of artificial diets and will increase weaning success (Ribeiro et al., 1999). Finally, possible optimum feed schedules will be discussed and a preliminary cost benefit analysis comparing alternative diets to *Artemia* will be undertaken.

This study is outlined as follows:

a) The effect of *Artemia* enrichment diets and ration on the growth and condition of juvenile pot-bellied seahorses (chapter 2).

b) The effect of alternative live and frozen diets on the growth and condition of juvenile pot-bellied seahorses, to determine the preferred prey size of seahorses, examine the relationship between predator and prey size and develop a weaning protocol (chapter 3).

c) Research into the histology and histochemistry of the development of the digestive tract of pot-bellied seahorses (chapter 4).

d) Research into the ontogenetic development of the digestive enzymes of pot-bellied seahorses (chapter 5).

e) Comparison of the costs and benefits of alternative live and frozen diets with *Artemia* (chapter 6).

CHAPTER TWO

EFFECT OF *ARTEMIA* ENRICHMENTS AND DAILY RATION ON THE GROWTH AND CONDITION OF POT-BELLIED SEAHORSES, *HIPPOCAMPUS ABDOMINALIS*.

2.1. INTRODUCTION

Pot-bellied seahorse culture is largely reliant upon enriched brine shrimp, *Artemia* as a food at most stages of rearing. *Artemia* is the most extensively used live prey species in the larviculture of other marine and freshwater fish and crustaceans (Kolkovski et al., 2000; Narcisso et al., 1999; Olsen et al., 2000; Planas & Cunha, 1999; Tamaru et al., 2002) mainly because *Artemia* can be stored for long periods of time as cysts and easily hatched under optimal culture conditions. A drawback of *Artemia* is that it is nutritionally deficient especially with respect to its fatty acid composition (Navarro et al., 1999; Tamaru et al., 2002; Watanabe et al., 1980), particularly the long chain omega-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) (Cho et al., 2001; Han et al., 2000; Narcisso et al., 1999; Navarro et al., 1999; Sorgeloos et al., 1991; Watanabe et al., 1980).

DHA and EPA are essential fatty acids (EFA) for marine fish species because larvae have little or no Δ5-desaturase activity, an enzyme needed to convert C-18 PUFAs to long chain HUFAs (Robin, 1995; Sargent et al., 1993). Although fish may be capable of converting EPA to DHA, the rate at which they are able to do so may be too low to satisfy the high DHA requirement (Sargent et al., 1997; Shields et al., 1999). The importance of these polyunsaturated fatty acids is beyond question, as marine fish require them for normal development (Izquierdo et al., 1989; Izquierdo, 1996; Kanazawa, 1985; Kitajima et al., 1980ab; Koven et al., 1989, 1990, 1992; Watanabe et al., 1983ab). The fatty acids are components of phospholipids, which are important sources of metabolic energy (adenosine triphosphate or ATP) (Tamaru et al., 2002) and are essential components of cell membranes of most tissues (Sargent et al., 1993; Tamaru et al., 2002). Fatty acids are also necessary for the development of the brain particularly the neural and visual functions of fish; they are the building blocks for many hormones and are important in maintaining membrane integrity and lipid transport (Bell et al., 1995; Cho et al., 2001; Koven et al., 2001; Kanazawa, 1993; Lie et al., 1992;

Sargent et al., 1993; Tamaru et al., 2002; Watanabe, 1993). Another important polyunsaturated fatty acid which is essential for some marine fish is the long chain omega-6 PUFA, arachidonic acid (AA 20:4n-6), the main precursor of eicosanoids responsible for osmoregulation, neural control and reproduction (Castell et al., 1994; Gapasin & Duray, 2001; Koven et al., 2001; Robin, 1995; Sargent et al., 1997). Sub-optimal supply of these three essential fatty acids may result in reduced viability, poor growth and high mortality of fish together with increased susceptibility to stress and disease (Koven et al., 2001; Olsen et al., 2000). Other essential dietary components (ie those which cannot be synthesized in sufficient quantities in the body to meet requirements) include amino acids, carbohydrates, phospholipids, minerals and vitamins (Cowey & Sargent, 1972; Gapasin & Duray, 2001; Koven et al., 2001; Rainuzzo et al., 1997; Tamaru et al., 2002).

In order to address the lack of polyunsaturated fatty acids in *Artemia* nauplii and ensure that fish are receiving their dietary requirements various enrichment techniques have been developed to enhance their nutritive value, especially their fatty acid content. *Artemia* are a passive non-selective filter feeder and this characteristic makes it possible to modify their nutritional profile by simply placing them into a medium, generally an emulsion, containing an abundant amount of DHA and relatively lower levels of EPA as fish are especially deficient in DHA and as *Artemia* convert DHA to EPA (Han et al., 2001; Koven et al., 2001; Navarro et al., 1999). This form of enrichment where the essential nutrients are packed within the digestive tract of *Artemia* has been termed bioencapsulation. By using different enrichment products or emulsions and different enrichment times the required concentration of fatty acids for a particular species may be achieved (Narcisso et al., 1999; Navarro et al., 1999). Examples of enrichment diets are unicellular algae (*Isochrysis*, *Tetraselmis*, *Chaetoceros*), baker's yeast, commercial emulsions (eg Selco™ and Algamac™ products, Frippack™, Instant microalgae™ pastes, Artemate™, Ika Omega™ and Acclimac™), emulsions prepared with special oils, thaustocytrids, microencapsulated diets,

fish byproducts and silages containing omega-3 PUFAs (Cho et al., 2001; Han et al., 2000; Narcisso et al., 1999; Tamaru et al., 2002). All of these enrichments differ in their lipid class composition, omega-3 polyunsaturated fatty acid content and DHA/EPA ratio which is used in many studies as an index of the optimal level of fatty acids required for normal growth and development (Gapasin & Duray, 2001).

The degree of success in modifying the nutritional profile of *Artemia* is variable and may be influenced by a number of factors. The base nutritional quality of *Artemia* can vary with its geographical origin (Han et al., 2000; Kreeger et al., 1991) while the type of enrichment diet used and enrichment conditions such as salinity, temperature, concentration of emulsion and duration of enrichment can influence the enrichment process. A particular problem with enrichment is that the biochemical composition of *Artemia* is not stable after enrichment so they must be on-fed to fish as soon as possible. However, as fish generally eat over longer periods of time, it will be necessary to re-enrich *Artemia* to ensure the dietary requirements of the fish are met (Han et al., 2000; Narcisso et al., 1999; Olsen et al., 2000). To achieve such re-enrichment *Artemia* may be flushed out from the recirculation system and new enriched *Artemia* introduced. In-tank green water cultures could also be used as a method of continuous enrichment in situ.

Another factor that determines the growth performance of fish is the quantity (ration) of food provided. As *Artemia* are expensive to culture effective feed management practices are required. Knowing the optimal ration or daily feeding rate is important not only for promoting the best growth and feed efficiency but also for economic and environmental reasons (Eroldogan et al., 2004; Linner & Brannas, 2001; Ng et al., 2000; Puvanendran et al., 2003; Webster et al., 2002). Specific growth rate, food conversion ratio and feed efficiency are the most important factors indicating the effectiveness of feed management and economic performance while the main factors closely associated with ration size are temperature and fish size (Rad et al., 2003).

Reducing feed wastage is important because it minimises water quality deterioration, decreases the cost of dietary nutrients and in relationship to *Artemia* reduces the loss of an expensive food source (Han et al., 2000; Langar & Guillaume, 1994; Puvanendran et al., 2003; Sumagaysay, 1998).

Findings from studies on the effect of enrichment diets on the growth and condition of seahorses are not conclusive with some authors reporting improved growth with enriched *Artemia,* while others found that different enrichment diets had no effect on the growth of seahorses (Chang & Southgate, 2001; Filleul, 1996; McEvoy et al., 1995; Sargent et al., 1997; Shapawi, 2001; Woods, 2003a). Interestingly it has also been noted that in some studies growth of seahorses fed unenriched *Artemia* was similar to those fed enriched *Artemia* (Wardley, 2001). Enrichment diets vary in their nutritional composition, for example, Algamac™ has 17.6% dry weight protein, 15.9% dry weight carbohydrate, 56.2% dry weight lipid, 2.88% EPA and 43.17% DHA; Protein Selco™ has 30% dry weight protein, 25% dry weight lipid, 1:1 EPA:DHA ratio; and a microalgae, *Tetraselmis* has 39% dry weight protein, 8% dry weight carbohydrate, 7% dry weight lipid, 5.3% EPA and trace amounts of DHA. Nichols (1999) reported that frozen juvenile seahorses contain 4.3% AA, 6.3% EPA, 12.2% DHA, sum omega-3 of 240 ± 34 mg/100g, 1.2% total lipid and 62.2% phospholipids. However the nutritional requirements of different aged seahorses is still largely unknown. Thus this study will determine whether the growth and condition of juvenile seahorses is influenced by enriching *Artemia* with one of a range of commercial enrichment products used in finfish aquaculture. The study will also determine the effect of a range of rations on the growth rate of juvenile seahorses as a further understanding of the quality and quantity of feed would improve the management of seahorses in culture. A biochemical analysis of the enriched *Artemia* would be beneficial however it is outside the scope of this study.

The general aims of this chapter are to 1) determine if *Artemia* enriched with one of a range of commercial enrichment diets has an effect on the growth and condition of seahorses and 2) determine the effect of a range of rations on the growth of seahorses. Specifically the study has the following aims:

Experiment One. Effect of *Artemia* enrichments on the growth and condition of pot-bellied seahorses.

a) To determine the most appropriate *Artemia* enrichment diet to use as a control diet to compare the performance (growth and survival) of other live feeds and frozen diets that could replace *Artemia*.

b) To determine the most appropriate *Artemia* enrichment diet for early juvenile seahorses in the event that they will not accept alternative feeds.

Experiment Two. Effect of different ration levels on the growth of pot-bellied seahorses.

a) To determine what dietary ration level gives rise to good growth in seahorses.

2.2. MATERIALS AND METHODS

2.2.1. EXPERIMENT ONE. EFFECT OF *ARTEMIA* ENRICHMENTS ON THE GROWTH AND CONDITION OF SEAHORSES

Seahorses were fed *Artemia* that had been enriched with one of a number of different enrichment diets to assess which of the *Artemia* enrichment diets tested promoted the best growth, condition and survival. The best diet was then used as a reference diet in all other feed trials. Enrichment diets used were chosen out of the range of enrichment diets that were readily available: Protein Selco™, Super Selco™, Algamac 3050™, mixed algae (*Tetraselmis, Isochrysis, Pavlova*) and Artemac™, which is an artificial *Artemia* replacement diet. The reference diet used was unenriched *Artemia*.

A total of 360 seven-week old pot-bellied seahorse juveniles were transferred from Seahorse World Pty. Ltd., Beauty Point, Tasmania to the climate controlled seahorse research room in the Aquaculture Centre, University of Tasmania, Launceston. Seahorses were treated prophylactically for protozoan infections of the skin with Acriflavine™ and 50 ppm formalin and distributed randomly to each tank (20 fish per tank). Each tank was randomly allotted a treatment (1 - 6, unenriched, Super Selco™, Protein Selco™, Algamac 3050™, Artemac™ and mixed algae) and replicate number (1 - 3).

System Configuration

Pot-bellied seahorses were maintained in eighteen 20 L natural coloured fibreglass (fawn coloured sides) conico-cylindrical tanks with a white gelcoat base within a recirculation system for 56 days. Seawater entered the tanks overhead at a flow rate of 1.7 - 2.2 L min^{-1} and left through a central mesh covered outlet, from which it flowed into an external stand pipe and returned through a biofilter to a reservoir (400 L) via a drain. The seawater was pumped around the system by a submersible pump. Each tank was fitted with holding substrates made of plastic mesh, and an airstone (Figure 2.1, 2.2, 2.3).

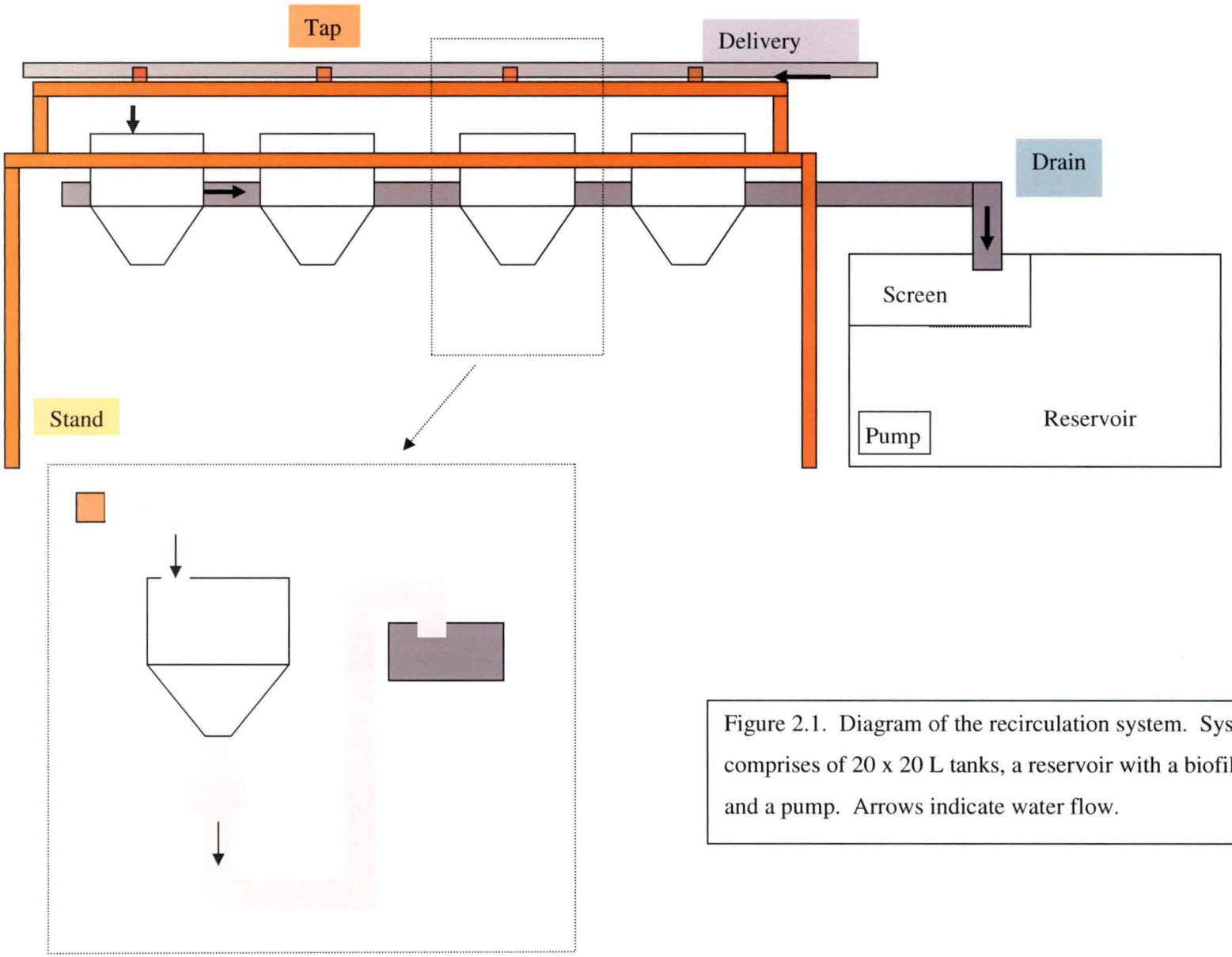


Figure 2.1. Diagram of the recirculation system. System comprises of 20 x 20 L tanks, a reservoir with a biofilter and a pump. Arrows indicate water flow.

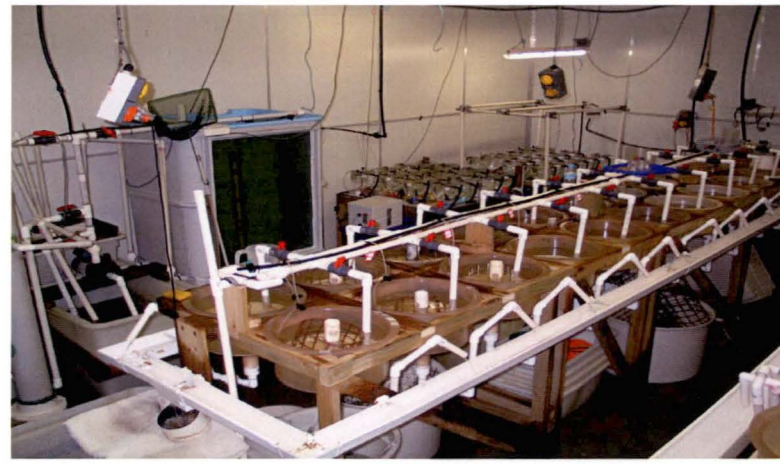


Figure 2.2. The recirculation system used for the feed trial. Includes 20 x 20 L tanks, a 400 L reservoir with biofilter, inflow system, external stand-pipes and a drain.

Figure 2.3. The recirculation system used for the feed trial. Includes 20 x 20 L tanks, a 400 L reservoir with biofilter, inflow system, external stand-pipes and a drain. There is a mesh holding substrate, airstone, inlet and outlet for each tank.

Tanks were cleaned daily by turning the water flow off and siphoning the detritus from the bottom of the tanks. The water in the reservoir was topped up and the holding substrates were washed down every day to maintain desirable water quality parameters. Feed (from the previous day) was removed from the system every morning when the tanks were flushed by placing larger screens on the central outlet and a smaller mesh screen overlying the dacron at the end of the drain. The system was treated in week 3 and week 6 with Acriflavine™ and 50 ppm formalin to reduce protozoan numbers and maintain the health of seahorses.

The seahorses were kept under a photoperiod of 12:12 LD (0800 - 2000 h light) with a light intensity of 1.2 - 2.1 umol.m^{-2}.s^{-1} (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters were: dissolved oxygen (7.2 - 7.5 mg/L), total ammonia (0 - 0.25 mg/L), nitrite (0 - 0.25 mg/L), nitrate (5 - 10 mg/L), pH (7.5 - 8.3) and temperature (18 ± 0.5°C).

Feed and Enrichment

Hatching *Artemia*

Prime™ *Artemia* cysts at a density of 2 g L^{-1} were hydrated in 25°C tap water for 3 hours. The hydrated cysts were placed in a 12.5% sodium hypochlorite solution at a concentration of 1 in 8 (hypochlorite:water) for 10 minutes or until the brown cysts had changed to orange in colour. Decapsulated cysts were collected on a 100 - 130 µm sieve and thoroughly washed with freshwater to remove chlorine.

Washed cysts were resuspended in 6 L of 25°C vigorously aerated seawater under bright light for hatching. Twenty-four hours after decapsulation the hatched nauplii were harvested using a 100 - 130 µm screen by turning off the aeration and applying a bright light to the bottom of the separation cone. Nauplii were washed with 25°C freshwater to remove contaminants and

transferred back into aerated acclimated seawater for ongrowing to the instar II stage.

Feed Rate and Enrichment Diets

Seahorses were fed a feed rate (dry feed) of 5% body weight (BW) day^{-1} (Thompson, 1999), calculated using data on the hatch rate, wet weight and dry weight of instar II *Artemia* (Appendix 9.1). The feed weight was recalculated after fish were weighed every 2 weeks and the ration was fed over 3 meals (0800 h, 1200 h, and 1700 h).

On reaching the instar II stage the *Artemia* were harvested, washed, resuspended in water and distributed evenly on the basis of volume between six 20 L containers, one for each enrichment treatment (Table 2.1). To reduce error in the distribution of *Artemia* the sequence was changed each day.

The enrichment diets were added at the manufacturers recommended application rate and time (Table 2.1, Appendix 9.2). *Artemia* were left to enrich overnight (15 hours) and in the morning were harvested, thoroughly washed with freshwater and returned to vigorously aerated seawater. Based on volume the morning feed was then collected and the remaining feed was re-enriched (Table 2.1) for the afternoon meals (further 4 and 9 hours enrichment).

To remove protozoan contaminants all feeds were treated with formalin (250 ppm) for half an hour prior to being washed and fed to the fish. For each treatment equal volumes of suspended *Artemia* were distributed to each replicate tank. To ensure the distribution of *Artemia* was equal among the three replicates in each treatment the tank sequence was changed every meal time.

Table 2.1. The *Artemia* enrichment diets fed to the pot-bellied seahorses and their required dose rates and enrichment times.

Treatment	1st Enrichment			Re-enrichment		
Enrichment Diet	Dose	Start time	End time	Dose	Start time	End time (2nd and 3rd feed)
Algamac 3050™	0.2 g L^{-1}	1600 h	0700 h	0.2 g L^{-1}	0800 h	1200 h / 1700 h
Super Selco™	600 mg L^{-1}	1600 h	0700 h	600 mg L^{-1}	0800 h	1200 h / 1700 h
Protein Selco™	600 mg L^{-1}	1600 h	0700 h	600 mg L^{-1}	0800 h	1200 h / 1700 h
Artemac™	0.4 g L^{-1}	1600 h	0700 h	0.2 g L^{-1}	0800 h	1200 h / 1700 h
Algae (*Isochrysis*, *Tetraselmis*, *Chaetoceros*)	5^3cell/ml	1600 h	0700 h	3^3cell/ml	0800 h	1200 h / 1700 h
Unenriched	-		-	-		-

(Refer to Appendix 9.3 & 9.4 for the nutritional profiles of *Artemia*, seahorses and *Artemia* enrichment diets)

Effect of Enrichment Diets on the growth of seahorses

The individual weight of seahorses in all treatments was measured in weeks 0, 2, 4, 6, and 8. The total length (back of crown to end of tail) of individual seahorses was measured at the beginning (week 0) and on completion (week 8) of the feed trial.

Specific growth rate of seahorses in each treatment was calculated using the equation:

$$SGR = 100.(\ln W_f - \ln W_i) / \Delta t$$

Where SGR is specific growth rate (% day^{-1}), W_f is the final weight (g) of seahorses over the time interval that weight was measured, W_i is the initial weight (g) of the seahorses, and Δt is the time (days) over which weight was measured.

Mortalities were removed from the tank when observed and recorded daily. In the enrichment diet trial 1.67% of the seahorses died (with 1 in the nil enrichment, 1 in the algae, 2 in the Super Selco™ and 2 in the Algamac 3050™ enrichment treatment). Mortalities were not replaced in this trial.

Statistical Analysis

All statistical analyses were carried out using the SPSS (version 11) software package. The statistical methods examined the effect of different *Artemia* enrichment diets on the growth and condition of seahorses.

Nested Analysis of Variance using the General Linear Model was used to compare the effect of different treatments on the weight of seahorses and Tukey's Honestly Significant difference (HSD) test was used to determine significance between means. The level of significance of $p < 0.05$ was used.

The condition index of seahorses was determined through the relationship between length and weight, which were calculated as the slopes of the least squares linear regression. Differences between treatments in each trial were

analysed using Analysis of Covariance (ANCOVA) (DeVlaming et al., 1982). Both weight and length data were log (χ + 1) transformed to approximate normal distribution and p values < 0.05, were considered significant. Size heterogeneity was expressed as the Coefficient of Variation (CV) of mean body weight and mean length.

2.2.2. Experiment Two. Effect of ration levels on the growth of pot-bellied seahorses

Seahorses were fed a range of daily rations to determine the optimum daily feed ration for good growth, condition and survival in the culture of pot-bellied seahorses. Further understanding of feeding in seahorses will help to improve food management.

A total of 360 seven-week old pot-bellied seahorses were transferred from Seahorse World Pty. Ltd. to the Aquaculture Centre and maintained experimentally as described in section 2.2.1. Seahorses were treated (Acriflavine™ and 50 ppm formalin) and distributed randomly to each tank (20 fish per tank). Each tank was randomly allotted a treatment (1 - 6, 1.25%, 2.5%, 3.75%, 5%, 6.25% and 7.5% body weight day^{-1}) and replicate number (1 - 3), giving a total of 18 tanks.

The seahorses were kept under a photoperiod of 12:12 L:D (0800 - 2200 h light) with a light intensity of 1.3 - 2.2 $umol.m^{-2}.s^{-1}$ (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters were: dissolved oxygen (7.2 - 7.7 mg L^{-1}), total ammonia (0 - 0.1 ppm), pH (7.4 - 8.4) and temperature (18 ± 0.5°C). The water in the system was treated in week 4 with Acriflavine™ and 50 ppm formalin to reduce protozoan numbers.

Feed

Artemia were hatched as per section 2.2.1 (Feed and Enrichment - Hatching *Artemia*) and were enriched with Algamac 3050™ using the dose rate and direction described in Experiment 1.

Seahorses were fed at feed rates of 25%, 50%, 75%, 100%, 125% and 150% with 100% being the 5% body weight day^{-1} reference ration equating to 1.25%, 2.5%, 3.75%, 5%, 6.25% and 7.5% body weight day^{-1} (dry weight food : wet weight fish basis). Feed rates were calculated using data on the hatch rate, wet weight and dry weight of instar II nauplii (Appendix 9.1) and feeds were recalculated every two weeks following the weight checks.

To ensure that the feed rates were met the *Artemia* required for each treatment were grown and enriched separately. *Artemia* on the basis of volume were fed out over two meals (0900 h and 1300 h) and to ensure that the distribution of *Artemia* was equal among the three replicates in each treatment the tank sequence was changed every feed time. Feeding frequency was set at two meals a day because, particularly at the lower rations, very low prey densities may have been produced if rations were divided over three meals.

To remove protozoan contaminants all feeds were treated with formalin (250 ppm) for half an hour prior to being washed and distributed to the fish.

Effect of ration levels on the growth of seahorses

The individual weight of seahorses in all treatments was measured in week 0, 2, 4, 6 and 8. The total length (back of crown to end of tail) of individual seahorses was measured at the beginning and on completion of the feed trial.

Specific growth rate of seahorses was measured as per section 2.2.1.

Food conversion ratio was calculated as

$$FCR = \text{total food added to tank} / \Delta \text{ weight}$$

(Waste feed levels were not measured in this trial).

Feed efficiency was calculated as

$$FE = (BW_f - BW_i) / \Sigma FW_t$$

Where BW_f is final body weight, BW_i is initial body weight and ΣFW_t is the total amount of food added to the tanks over the trial.

Mortalities were removed from the tanks when observed and recorded daily. In the trial 5.27% of the seahorses died (with 10 in the 1.25% body weight day^{-1} ration, 3 in the 2.5 % body weight day^{-1} ration, 2 in the 3.75 % body weight day^{-1} ration, 2 in the 6.25% body weight day^{-1} ration and 2 in the 7.5% body weight day^{-1} ration). Mortalities were not replaced in this trial.

The difference in weight of the seven-week old seahorses used in Experiment One and Two was due to the stock seahorses were collected from. When the experiment was run there was limited stock to select from at the time because of low recruitment in the system over a couple of months.

Statistical analysis

Statistical analysis was carried out using the SPSS (version 11) software package. The statistical methods describe the effect of different ration levels on the growth and condition of the seahorses.

The effect of different treatments on the weight of seahorses and the condition index of seahorses was determined as described in section 2.2.1.

Nested Analysis of Variance using the General Linear Model was used to compare the survival of seahorses in the different ration treatments and Tukey's Honestly Significant Difference (HSD) test was used to determine significance between means. The level of significance of $p < 0.05$ was used.

2.3 RESULTS

2.3.1. EXPERIMENT ONE. EFFECT OF *ARTEMIA* ENRICHMENTS ON THE GROWTH AND CONDITION OF POT-BELLIED SEAHORSES

There was no significant difference ($F = 0.671$, df 5, $p > 0.05$) between the weight of pot-bellied seahorses in the nil enrichment, Algamac™, Super Selco™, Protein Selco™, Artemac™ and algae treatments at the beginning of the feed trial (Figure 2.4, Appendix 9.5). At the completion of the trial there was no significant difference between the weight of seahorses fed different *Artemia* enrichments ($F = 1.414$, df 5, $p > 0.05$) (Figure 2.4, Appendix 9.5). Interestingly, the growth of seahorses fed the unenriched diet was not significantly different from the enriched diets.

Specific growth rates of seahorses fed *Artemia* enriched with nil enrichment, algae, Super Selco™, Protein Selco™, Artemac™, and Algamac™ from the beginning to end of the feed trial were 1.590%d^{-1}, 1.537%d^{-1}, 1.311%d^{-1}, 1.516%d^{-1}, 1.726%d^{-1} and 1.757%d^{-1} respectively.

The condition index of seahorses in the feed trial was determined by examining length to weight relationships. There was no significant difference ($F = 1.637$, df 5, $p > 0.05$) between the mean condition index of seahorses in different dietary treatments (Table 2.2).

There was little to no variation between the weights and lengths of seahorses in the unenriched, algae, Super Selco™, Protein Selco™, Artemac™ and Algamac 3050™ treatments in the diet trial (Table 2.2).

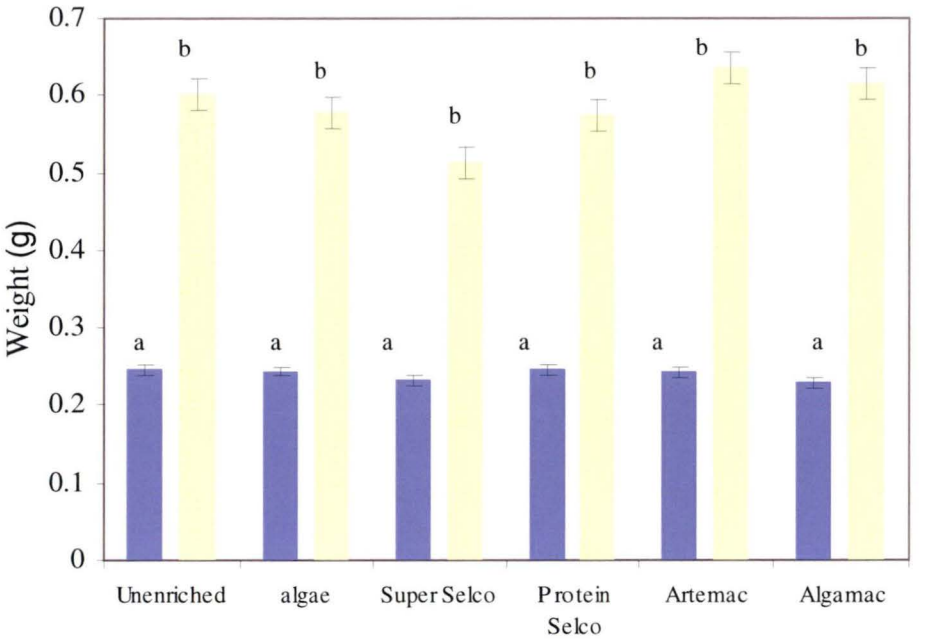


Figure 2.4. Initial ♦ and final ♦ mean (± S.E) weight of the pot-bellied seahorses at the beginning and end of the enrichment trial for each enrichment diet tested. Means which did not differ significantly in Tukey's HSD tests share the same common superscript. (Refer to Appendix 9.5 for further information on mean initial and final weights of seahorses).

Table 2.2. Effect of enrichment diets on the length to weight relationship (condition index) of the pot-bellied seahorses and the start and end coefficient of variation for weight and length for each treatment (a = elevation of the line; b = slope of the line, lower slope = poorer condition; r^2 = explanatory variable).

Enrichment	Condition Factor				Coefficient of Variation			
					Weight	Weight	Length	Length
	a	b	r^2	p	Initial	Final	Initial	Final
Unenriched	-1.930	1.124	0.804	>0.05	0.244	0.367	0.059	0.105
Algae	-1.802	1.057	0.836	>0.05	0.194	0.324	0.054	0.101
Super Selco™	-1.796	1.046	0.846	>0.05	0.239	0.370	0.065	0.106
Protein Selco™	-1.791	1.052	0.776	>0.05	0.269	0.322	0.071	0.096
Artemac™	-2.235	1.285	0.895	>0.05	0.247	0.336	0.061	0.096
Algamac 3050™	-1.942	1.134	0.723	>0.05	0.182	0.320	0.058	0.089

(Regression equation: $\log_{(\chi+1)} = b \log_{(\chi+1)}$ length + $\log_{(\chi+1)}$ a)

2.3.2. Experiment two. Effect of ration level on the growth of seahorses

There was no significant difference ($F = 0.706$, df 5, $p > 0.05$) between the weight of pot-bellied seahorses in the different ration treatments at the beginning of the ration trial (Figure 2.5, Appendix 9.6). At the completion of the trial there was a significant difference between the weight of seahorses fed different rations ($F = 0.790$, df 5, $p < 0.05$) (Figure 2.5, Appendix 9.6). Weight tended to increase with increasing ration with some levelling at higher rations. It was also found that significantly ($F = 7.3$, df 5, $p < 0.05$) more seahorses died in the lowest ration treatment.

Specific growth rates of seahorses fed a dietary ration of 1.25%, 2.5%, 3.75%, 5%, 6.25% and 7.5% body weight day^{-1} from the beginning to the end of the feed trial were 2.0014%d^{-1}, 2.2566% d^{-1}, 2.3617% d^{-1}, 2.7451% d^{-1}, 3.0822% d^{-1} and 3.1207% d^{-1} respectively.

The condition index of seahorses in the ration trial was determined by examining length to weight relationships. A significant difference ($F = 5.780$, df 5, $p < 0.05$) was found between the mean condition index of seahorses in different ration treatments, with seahorses fed a ration of 1.25% and 2.5% body weight day^{-1} having poorer condition (lower slope of 0.446 and 0.460 respectively) compared with all others (Table 2.3).

The results also show that at the end of the trial there was variation between the weights of seahorses in the different treatments, with seahorses in the 6.25% body weight day^{-1} ration having a greater spread of weights than seahorses in the other treatments. There was little to no variation between the start weights and lengths for seahorses fed different dietary rations (Table 2.3).

The food conversion ratios for the 1.25%, 2.5%, 3.75%, 5%, 6.25%, and 7.5% body weight day^{-1} rations were 0.896:1, 1.53:1, 2.72:1, 2.32:1, 3.04:1, and

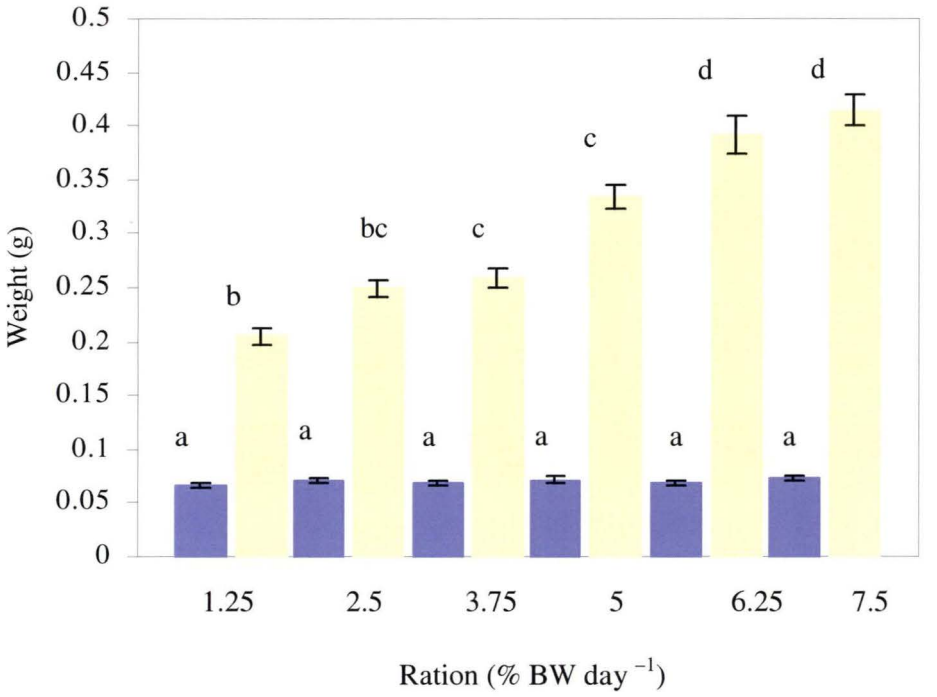


Figure 2.5. Initial ♦ and final ♦ mean (± S.E) weight of pot-bellied seahorses in each treatment of the ration trial. Means which did not differ significantly in Tukey's HSD test share the same common superscript. (Refer to Appendix 9.6 for further information on mean initial and final weights of seahorses).

Table 2.3. Effect of ration size on the length to weight relationship (condition index) of the pot-bellied seahorses and the start and end coefficient of variation for weight and length for each treatment (a = elevation of the line; b = slope of the line, lower slope = poorer condition; r^2 = explanatory variable).

Ration	Condition Index				Coefficient of Variation			
					Weight		Length	
	a	b	r^2	p	Initial	Final	Initial	Final
1.25% bwd^{-1}	-0.674	0.446	0.668	<0.05	0.2620	0.2694	0.0800	0.0849
2.5% bwd^{-1}	-0.692	0.460	0.601	<0.05	0.2719	0.2331	0.0730	0.0779
3.75% bwd^{-1}	-0.817	0.532	0.629	<0.05	0.2470	0.2440	0.0726	0.0741
5% bwd^{-1}	-0.957	0.620	0.747	<0.05	0.2657	0.2696	0.0692	0.0928
6.25% bwd^{-1}	-1.269	0.799	0.754	<0.05	0.2603	0.3355	0.0774	0.1012
7.5% bwd^{-1}	-1.048	0.675	0.580	<0.05	0.2500	0.2713	0.0694	0.0880

(Regression equation: $\log_{(\chi+1)}$ weight = b $\log_{(\chi+1)}$ length + $\log_{(\chi+1)}$ a)

3.53:1 respectively. The feed efficiency for the 1.25%, 2.5%, 3.75%, 5%, 6.25%, and 7.5% body weight day^{-1} rations were 1.11, 0.64, 0.44, 0.43, 0.32, and 0.28 respectively.

2.4. DISCUSSION

A range of enrichment diets differing in lipid and protein content were used to determine which *Artemia* enrichment promoted the best growth in juvenile pot-bellied seahorses. The results suggest that seahorses fed *Artemia* enriched with Super Selco™, Protein Selco™, Algamac 3050™, Artemate™, or mixed algae showed no significant statistical difference in growth or condition index. It was also found that there was no significant difference in growth of seahorses fed enriched *Artemia* and seahorses fed unenriched *Artemia*.

These results suggest that in relation to growth costly enrichment diets are not really required. However in larval rearing of other marine fish enriching the live prey with essential fatty acids, DHA and EPA have resulted in better growth and survival while other studies have shown that growth is similar in fish fed enriched and unenriched prey. Job et al. (2002) found that when *Hippocampus kuda* were fed *Artemia* enriched with a crustacean based (*Acetes* sp.) enrichment product and a fish based enrichment product there was no difference between growth in standard length and weight. However, they also noted that survival to market size was twice as high in seahorses fed *Artemia* enriched with the *Acetes* sp. product.

Although enrichment diets may show no effect on the growth and survival of fish in the short term they do show an effect in the long term. As mentioned above, seahorses fed *Artemia* enriched with *Acetes* sp. had a higher survival rate than those fed a fish based enrichment diet. Gapasin and Duray (2001) found that milkfish (*Chanos chanos*) fed DHA enriched food during its larval stage showed significantly better growth in the nursery stage than larvae that had been fed unenriched diets. Ako et al. (1994), Dhert et al. (1990) and Kraul et al. (1993) also found that there was no obvious differences in the

survival and growth of fish fed enriched *Artemia* over the short term but noted that fish fed enriched *Artemia* were stronger and not as susceptible to handling and stress as those fed unenriched *Artemia*. It can be suggested that although fish fed enriched *Artemia* may show no marked improvements in growth *Artemia* enrichments should be used as they have been shown to significantly improve the general health and well being of fish. In this study it was found that the liver of seahorses in the nil enrichment treatment was in poorer condition (dark orange in colour, had become fatty and were shapeless) than those seahorses fed enriched *Artemia* thus making *Artemia* enrichment diets essential.

Wong and Benzie (2003) found that the growth rate of *Hippocampus whitei* was consistently higher in the treatments fed enriched diets but there was no difference between the condition of seahorses fed unenriched and enriched diets. Chang and Southgate (2001) found that there were marked improvements in the growth and survival of *H. kuda* fed enriched *Artemia* and that a higher level of DHA is required for optimal growth of juveniles from 3 to 33 days old. In the present study an effect on the growth may have been seen if younger seahorses were studied, as essential fatty acids are shown to be especially important in the early stages of other marine fish and juvenile seahorses at different ages may have different fatty acid requirements (Wong & Benzie, 2003). The effect of *Artemia* enrichments may also have been affected by the strain of *Artemia* used and their developmental stage, prior nutritional status of the animals and the enrichment diet (type of emulsion, enrichment conditions and enrichment time) (Han et al., 2000; Narcisso et al., 1999; Navarro et al., 1999). The dose rate of enrichments can also alter the effect of enrichment as excess HUFA levels have been shown to have negative effects on the growth of fish (Corazze, 2001; Kolkovski et al., 2000). Ration could also have had an affect. In this study a 5% body weight day^{-1} ration was fed and if this was below optimum, growth differences could have been masked by sub-optimal feed intake.

In addition to the effect that the nutritional value of prey has on growth, the quantity of feed (ration) can also affect the growth rate of fish (Paul et al., 1994; Stead et al., 1996). It is important to find the optimum ration size, which has been defined as the one that gives the best growth and feed conversion ratio (FCR) (Silvia and Anderson, 1995; Rad et al., 2003). When looking at ration curves there are maintenance, optimum and maximum ration requirements for fish. The relationship between ration size and growth rate has been shown to be positive and generally a linear increase in specific growth rate (SGR) is seen with increasing feed rates. At rations below the maintenance level fish lose weight and there is greater size variability among the fish. As food availability increases growth is accelerated until the maximum ration requirement (ie maximum amount of food fish are capable of taking up) is met. At ration levels higher than the maximum requirement fish growth is negligible and feed is wasted (Johnston et al., 2003; Khan et al., 2004). The optimum feed ration therefore is when maximum growth is achieved with maximum feed efficiency, which occurs at a level below satiation (Rad et al., 2003; Zakes et al., 2003).

Studies with several fish species have shown that with increasing feeding rate, the growth increases at higher ration levels and decreases at lower ration levels. Khan et al. (2004) found that growth of the Indian major carp (*Cirrhinus mrigala*) fingerlings improved with increasing ration levels up to 6% body weight day^{-1} however better feed efficiency was achieved at a feed rate of 4% body weight day^{-1} thus indicating that feeding fish in the range of 4 - 6% body weight day^{-1} results in maximum utilisation of food for growth. Khan et al. (2004) also found that fish fed 2% body weight day^{-1} had poor growth and it is suggested that this ration size approximates the maintenance requirements. Rad et al. (2003) found that the optimum feeding rate for Siberian sturgeon (*Acipenser baerii baerii*) was between 1% and 1.25% body weight day^{-1}. The mean values of SGR obtained at these feeding rates are close to one another, while the FCR of sturgeons fed at 1% body weight day^{-1} was better. Since a similar growth rate is achieved under both feeding rates,

feeding sturgeons at 1% is more likely to be the optimum point as far as economic considerations are concerned. Feeding large sturgeons above or below 1% body weight day^{-1} does not favour either the growth or food conversion ratio as sturgeons fed at rates of 0.75% body weight day^{-1} and 1.5% body weight day^{-1} showed lower growth rates and poorer food conversion ratios. The significant increase seen in the SGR of fish fed the higher feeding rates (1% and 1.25%) suggest that a large portion of dietary nutrients have gone into growth rather than maintenance while the suppressed growth in sturgeons fed 0.75% body weight day^{-1} ration suggest that the bulk of nutrients were used for maintenance. Reduced growth at 1.5% ration can be explained by a reduction in the retention of dietary nutrients when feed intake was high.

Puvanendran et al. (2003) found that juvenile yellowtail flounder (*Limanda ferruginea*) fed with a 3% body weight day^{-1} ration were significantly larger and heavier in terms of size and weight than fish fed with 1%, 1.5%, or 2% body weight day^{-1} rations. However, juvenile fish fed with 3% ration showed a significantly higher FCR than the fish fed with lower rations, which indicates greater food wastage. The study also showed that maximum food conversion efficiency (lower FCR) occurs at ration levels below those at which maximum growth occurs with the fish being fed a 3% body weight day^{-1} ration having maximum growth and those fed with a 1% body weight day^{-1} ration showing the maximum food conversion efficiency. Taking growth and feed efficiency into consideration, there are a range of possible feeding levels for the pot-bellied seahorse, the choice of which depends on whether maximum growth, optimal food conversion, or a balance between the two is sought.

In the present study it was found that growth of the pot-bellied seahorse increased significantly with increasing ration size. If maximum growth were to be used as an indicator of the optimum ration then the optimum ration for pot-bellied seahorses would be in the vicinity of 6.25 - 7.5% body weight day^{-1}

[1]. The maintenance ration for seahorses was not determined in this ration trial. However it could be suggested that a feed rate of 1.25% body weight day^{-1} could be close to the maintenance ration of 15 week old seahorses because it was found that their growth and condition was poorer than seahorses fed the higher rations and this treatment group suffered significantly more mortalities. If efficient food conversion was to be used as an indicator of optimum ration level then the lower ration levels would be chosen as they have lower food conversion ratios (FCR) and an FCR rate near 1 is the optimal level for cultured fish fed artificial diets. However, it was found that the condition of the seahorses fed the lower rations (1.25% and 2.5% body weight day^{-1}) was significantly lower than the seahorses fed the other rations studied. If maximum growth was used an indicator for optimum ration levels then the higher ration levels would be chosen but they have high FCR values of 3 and 3.5 which indicate significant food wastage. It could be suggested, therefore, that an optimum ration level for seahorses is a balance between feed efficiency and maximum growth. To gain a better understanding of feed efficiency of seahorses fed different rations it would have been more accurate to calculate FCR based on actual intake. However collection of waste feed proved to be difficult hence total feed added was used to calculate food conversion ratios. During further studies ways of measuring waste feed were developed which allowed future FCRs to be based on actual intake rather than total food added.

Ration size is influenced by fish age and size (Khan et al., 2004; Paul et al., 1994). For example Puvanendran et al. (2003) and Eroldogan et al. (2004) found that smaller yellow tail (*Ocyurus chrysurus*) juveniles and European sea bass (*Dicentrarchus labrax*) respectively require a higher ration levels in terms of percent body weight to achieve optimal growth than larger juveniles, which require smaller rations. Other factors that may have had an influence on feed intake are experimental conditions such as stocking rate, water quality, water flow rate, shape of the tank, the diet and the feeding regime (Eroldogan et al., 2004; Rad et al., 2003; Webster, 2002). Johnston et al.

(2003) found that the effect of ration size on growth depended on the feeding frequency with the optimal ration for fish fed once a day being between 6 - 8% body weight day^{-1}; fish fed twice a day showing better growth when fed rations between 8 - 12% body weight day^{-1}, and fish fed 3 times a day showing similar growth for all rations fed except the lowest ration where fish grew the slowest. Taking these influences on daily ration into account it could be that the ration curve seen in this experiment may have been different if younger seahorses and different feeding rates had been studied. Another factor that may affect feed intake is prey density. Although *Artemia* were present in all tanks at the end of the day (even in the lowest ration treatment) it could be suggested that feed rates were adequate. However the reduced growth rates seen in the lower ration treatments could be due to the density of *Artemia* being below the optimum feeding density of seahorses.

In conclusion it was found that different *Artemia* enrichments had little effect on the growth or condition of juvenile seahorses and that growth and condition of seahorses fed enriched *Artemia* was similar to those fed unenriched *Artemia*. It is suggested however that *Artemia* are enriched as enrichment diets ensure the well-being and health of seahorses. It was also found that of the rations tested a 6.25 - 7.5% body weight day^{-1} ration provided the better growth for seahorses. These higher ration levels displayed higher FCR values compared to seahorses fed lower rations and better growth rates accompanying higher FCR values are usually indicators of food wastage through overfeeding. Thus considering the growth and the fact that although seahorses fed the lowest rations (1.25% and 2.5% body weight day^{-1}) had better food conversion ratios, they showed significantly poorer condition suggesting that a 5% body weight day^{-1} ration would be appropriate for seahorses. However further work is required on the maintenance, optimum and maximum ration requirements of seahorses if cost effective feeding programs are to be further refined.

CHAPTER THREE

EFFECT OF ALTERNATIVE LIVE AND FROZEN DIETS ON THE GROWTH AND CONDTION OF POT-BELLIED SEAHORSES

3.1. INTRODUCTION

During culture conditions seahorses are reliant initially on instar II *Artemia* and are then fed frozen diets from about 4 to 6 months onward. *Artemia* are widely used as a live feed in marine aquaculture because they are available as dormant cysts, simple to hatch and their biology allows for enrichment of required nutrients. However they are unreliable in terms of sustained production, expensive and time consuming to culture due to equipment required and intensive labour. In addition, cyst quality can be seasonally dependent (in the wild), their nutritional quality is highly variable, they are a potential source of contagious pathogens and, as seahorses increase in size, it is necessary to ongrow *Artemia* which increases production costs (Baskerville-Bridges & Kling, 2000a; Borlondan et al., 2000; Domingues et al., 2000; Gordon et al., 1998; Koven et al., 2001; Leu & Liou, 1992; Petkam & Moodie, 2001; Woods & Valentine, 2003). For the culture of pot-bellied seahorses to be economically feasible there is a need for less expensive alternative diets to totally or at least partially replace *Artemia* in their diet.

Ultimately, the preferred alternative diet would be an artificial diet which is economical to produce, has a relatively long shelf life, is easy to dispense and has a formulated nutritional quality (Baskerville-Bridges & Kling, 2000a; Borlondan et al., 2000; Hamlin & Kling, 2001). Introducing artificial diets can be difficult as a formulated diet only becomes acceptable when it attracts and satisfies the requirements of fish. Factors of importance include physical (buoyancy, movement, appearance, feel/texture), chemical (attractants), and nutritional (digestible, nutritionally complete) attributes of the diet and knowledge of the fishes digestive development (Baskerville-Bridges & Kling, 2000b; Gordon et al., 1998; Guthrie et al., 2000). Before an artificial diet can be developed for seahorses much work is required on the specific nutrient (lipids, phospholipids, essential fatty acids, protein and amino acids) requirements of fish of different ages, palatability of a diet, physical characteristics (structure) of the diet, feeding

behaviour of the seahorses or distribution of the diet (ie sink slowly or remain slightly buoyant), ability of the fish to ingest the diet, digestive capabilities (ie onset of digestive enzymes) and mechanisms of absorption (Borlondan et al., 2000; Cahu & Infante, 2001; Coutteau et al., 1996; Hart & Purser, 1996; Khemis et al., 2003; Koven et al., 2001; Petkam & Moodie, 2001; Rosenlund et al., 1997; Teshima et al., 2000; Yufera et al., 2000). It is also important to determine the appropriate size of the diet and to determine the time a fish needs to recognize and accept a diet (Cahu & Infante, 2001; Koven et al., 2001).

ALTERNATIVE LIVE DIETS

Due to a lack of knowledge on the food requirements of seahorses and the limited success in experimental trials (Wilson, 2003; Woods, 2003a; Woods & Valentine, 2003) the development of an artificial diet formulated specifically for seahorses is not feasible at this time. Instead possible alternative diets to reduce the reliance on *Artemia* in the culture of seahorses may include other natural diets in either live or frozen form. Wild seahorses are known to eat predominantly crustaceans (shrimp, crabs, amphipods, mysids, euphasids), small fish and worms and in culture they have been successfully fed copepods, mysids and amphipods (Payne & Rippingale, 1997; Woods, 2000ab). While it may seem attractive to collect wild food, it may be unreliable and dependence on unreliable wild stock may limit seahorse production. In addition, collection may be restricted by legislation (Callan et al., 2003; Woods & Valentine, 2003). However the use of wild food to partially replace *Artemia* in the diet of seahorses may decrease the cost (by reducing the number of live feed cultures) of feeding juvenile seahorses. A further method to reduce the cost could be to wean seahorses onto frozen diets at the earliest development stage possible. At present in culture they are introduced to frozen mysids (*Paramesodopsis* rufa, *Tenagomysis* sp.) at around six months of age (Seahorse World Pty. Ltd. diet). In older fish, Woods and Valentine (2003) found that 10-month old seahorses fed frozen mysids, *Amblyops kempi* had similar growth to those fed enriched *Artemia*.

Seahorses are generally visual "sit and wait" ambush predators using vegetation for camouflage but in the absence of physical structures they actively pursue their prey. Unlike most other teleost fish, syngnathids (which include seahorses, pipe fish and sea dragons) are unable to protrude their mouths to aid in prey capture because their jaws are fused and their small mouth is located at the end of an elongated tubular snout. This pipette-like feeding structure has allowed seahorses to specialise in suction feeding (Bergert & Wainwright, 1997). Seahorses, although extremely manouverable within vegetation, are constrained by their limited swimming capability and can not rapidly pursue or 'lunge' at their prey (Domenici & Blake, 1997). As a consequence they wait until they are within striking distance (swim up to or extend the body out from substrate while holding on with their prehensile tail) and capture their prey with a rapid vertical swing of the head to bring the mouth close to the prey so that it is drawn into the buccal cavity by suction (inhalant current caused by expansion of the buccal cavity) (Ocken, 1994; Muller & Osse, 1994). A long snout aids in feeding as it increases the distance from where the fish can initiate an attack and allow for a faster vertical head flick, which would enable seahorses to eat relatively fast moving prey (Ocken, 1994). While large juveniles and adult seahorses display both pelagic and benthic behaviour, newborns to about 4 weeks old tend to actively swim and feed in the water column. Newborns tend to link tails or attach to floating substrates and this may be used in the wild as a dispersal mechanism as well as positioning them amongst zooplankton (Kanou & Kohno, 2001, Woods, 2000ab).

Prey selection can be a passive process or it can involve active predator choice. Passive selection depends primarily on encounter rates and actual capture success of different prey types and does not involve any active choice from the predator. A major determinant of encounter rate or capture success is size and it is often found that capture success is strongly and inversely related to prey size (Christensen, 1996; Reimchem, 1991) which can bring about selection of smaller

than average prey sizes which are easier to handle. Active selection, on the other hand, occurs when fish choose upon encountering prey whether to attack the prey or not. This form of selection requires that the predator is capable of taking into account pre- or post-capture constraints such as the time needed to manipulate and ingest different sizes of prey (Turesson et al., 2002). Some studies have found that fish selectively feed on relatively large prey as feeding on larger prey ensures the energy demand of fish is met and growth rates are enhanced (Gerking, 1994; Keeley & Grant, 2001; Schabetsberger et al., 2003; Utne-Palm, 1999; Viitasalo et al., 2001). In contrast other studies on size selective predation have shown that when given a choice fish feed on sizes of prey that are smaller than their maximum ingestible size (Juanes & Domenici, 1994; Nilsson & Bronmark, 2000; Wahl & Stein, 1988; Webb, 1986). The relationship between predator (total length, gape or jaw length) and optimal prey size therefore appears highly species-specific. This study will determine the preferred prey sizes of different aged seahorses and the preferred prey type of seahorses by using the selective index of Cheeson (1978, 1983).

A number of factors are known to influence prey selection and these include prey characteristics such as size of the prey (length, width, depth), their population densities, their motion (swimming ability), colour, sensory performance and spatial and temporal patterns (Domenici, 2001; Domenici & Blake, 1993ab; Moore & Moore, 1976; O'Brien, 1979; Reiriz et al., 1998). Prey behaviour, for example their foraging behaviour, or an activity pattern in response to a predator may also affect selection (Braband & Faafeng, 1993; Domenici, 2002; Domenici & Blake, 1991; Petterson & Bronmark, 1993; Turner & Mittelbach, 1990). A number of studies have reported that prey size increased significantly according to the size and age characteristics of the predator (Keskinen & Marjomarki, 2004; Pederson, 1999; Schabetsberger et al., 2003). The predators' feeding behaviour, sensory capabilities, prior experience, locomotor ability, and mouth dimensions (jaw length and gape) determines which prey are more likely to be detected and

captured (Blaxter, 1988; Cox & Pankhurst, 2000; Godin, 1978; Swenson & McCray, 1996; Werner et al., 1981). Other factors, which may affect prey selection, are environmental factors such as light intensity, water turbidity, structure density and temperature (Cobcroft et al., 2001; Furnass, 1979; Mills et al., 1986; Yin & Blaxter, 1987). An understanding of the factors, which are involved in live prey selection and consumption is essential for effective live feed management in intensive aquaculture of marine fish however an extensive examination of these factors are outside the scope of this study.

WEANING SEAHORSES ON TO FROZEN DIETS

In addition to prey size, time taken to recognise a new or potential prey influences the selection of a new food. During culture most marine fish species are transferred from a live diet to a dry diet; this transition is referred to as weaning. Le Ruyet (1999) described three weaning strategies: 1) wean at first feeding, 2) rear fish on live feed for some time and then replace live feed with a new diet abruptly, or 3) replace feed gradually over several days. The weaning strategy used in most hatcheries is to gradually diminish the amount of live feed and gradually increase the amount of formulated diet (Bengston et al., 1999). This weaning strategy may be favoured as the combined feeding of two diets, referred to as co-feeding, may pre-condition fish to more readily accept the new diet when the old diet is withdrawn (Rosenlund et al., 1997).

Weaning success can be affected by the duration of the change over period as time required to establish successful weaning is variable. For example Bengston et al. (1999) reported that winter flounder (*Pleuronectes americanus*) could be weaned in 1 week but other studies on flounder found that 12 to 20 days of weaning was required. It can take 1 to 11 days for 50% of turbot (*Scophthalamus maximus*) in different batches to establish feeding on new diets (Bromley & Howell, 1983). When weaning southern flounder (*Paralichthys lethostigma*), flounder can not be weaned onto artificial diets before 20 days if live feed is

abruptly ceased. However this period can be shortened when gradual weaning is employed (Daniels & Hodson, 1999) and greenback flounder (*Rhombosolea tapirina*) have been shown to successfully feed on a novel prey after 1 to 5 days exposure to the prey (Cox & Pankhurst, 2000). All these studies have indicated that a substantial overlap is required during weaning.

This study will determine whether the growth and survival of seahorses fed copepods, biofouling crustaceans, frozen mysids and frozen amphipods are similar to those fed enriched *Artemia*. It will also determine the relationship between fish size and prey size with prey width being studied, as many studies deem prey width as the primary determinant of prey selection (Cunha & Planas, 1999) and the preferred prey size and prey type of seahorses will be examined. The time required for a seahorse to accept a new diet will be determined and a weaning protocol for seahorses will be developed.

The general aims of this chapter are to 1) determine if growth and condition of seahorses fed alternative live diets is similar to seahorses fed enriched *Artemia*, 2) determine the relationship between predator and prey size and determine the preferred prey type and prey size of seahorses and 3) determine if the growth and condition of seahorses fed frozen diets is similar to seahorses fed enriched *Artemia* and develop a workable weaning protocol for pot-bellied seahorses. Specifically the study has the following aims:

Experiment One. Effect of biofouling crustaceans on the growth and condition of seahorses.

a) To determine whether the growth, condition and survival of 17-week old seahorses fed biofouling crustaceans is similar to seahorses fed enriched *Artemia*.

Experiment Two. Predator prey relationship and preferred prey type and size of seahorses.

a) To determine the preferred prey type and size of different aged pot-bellied seahorses;
b) To assess the relationship between seahorse length, weight, gape, snout length and eye diameter;
c) To determine whether there is a relationship between predator and prey size.

Experiment Three. Effect of copepods on the growth and condition of seahorses.

a) To determine whether the growth, condition and survival of 3-week old seahorses fed copepods is similar to seahorses fed enriched *Artemia.*

Experiment Four. Effect of frozen diets on the growth and condition of seahorses and the development of a weaning protocol.

a) To determine whether the growth, condition and survival of 13-week old seahorses fed frozen amphipods is similar to seahorses fed enriched *Artemia*;
b) To determine whether the growth, condition and survival of 13-week old seahorses fed frozen mysids is similar to seahorses fed enriched *Artemia*;
c) To determine whether frozen amphipods or frozen mysids give the best growth, condition and survival in pot- bellied seahorses;
d) To determine the time required for pot-bellied seahorses to accept a new diet.

3.2 Materials and Methods

3.2.1. Experiment one. Effect of biofouling crustaceans on the growth and condition of seahorses

The aim of experiment one is to determine if the growth, condition and survival of pot-bellied seahorses fed biofouling crustaceans was similar to seahorses fed enriched *Artemia*. Biofouling crustaceans included a variety of amphipods that were collected on specially built net panels.

This experiment was conducted in the Aquaculture Centre at the University of Tasmania, Launceston. A total of 120 seventeen-week old pot-bellied seahorses were randomly selected from seahorses held in the Aquaculture Centre. Seahorses were treated with Acriflavine™ and 50 ppm formalin for 24 hours to remove protozoans and distributed randomly to each of 8 tanks (15 fish per tank). Each tank was randomly allotted a treatment (1 - 2, biofouling or *Artemia*) and replicate number (1 - 4).

System configuration

Pot-bellied seahorses were maintained in eight 20 L conico-cylindrical tanks within a recirculation system described in section 2.2.1. The flow rate in each tank was maintained at an average of 2.5 L min^{-1}.

Tanks were cleaned daily (morning) by turning off the water flow and siphoning the detritus from the bottom of the tanks. The water in the reservoir was changed every two weeks and the holding substrates and mesh screens, which were placed on the stand pipes, were thoroughly washed every day to maintain desirable water quality parameters. Feed was removed from the system every night, when the tanks were siphoned and flushed by placing larger screens on the central outlet (stand pipe) and a smaller mesh screen onto which the *Artemia* collected at the end of the outlet drain. To ensure no *Artemia* escaped into the system and into the biofouling tanks the small mesh screens were returned to the stand pipes as

soon as flushing was complete, retaining the *Artemia* within the appropriate treatment tanks.

The seahorses were maintained under a photoperiod of 12:12 L:D (0800 - 2000 h light) with a light intensity of 1.3 - 2.2 umol.m^{-2}.s^{-1} (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters during the trial were: salinity (32 ± 2 ppt); temperature (18 ± 0.5°C); total ammonia (0 - 0.25 mg/L); nitrite (0 - 0.25 mg/L); nitrate (5 - 10 mg/L); pH (7.8 - 8).

Feed

Artemia were hatched as per section 2.2.1. and were enriched with Algamac 3050™ for 15 hours at a dose rate of 0.2 g L^{-1} for the morning feed and a further 4 hours (at the same enrichment dose rate) for the afternoon feed.

Biofouling crustaceans were collected on net panels, which were constructed from 15 mm electrical conduit pipe in the dimension of 20 cm in length by 15 cm in width; a white, 5 mm diameter, tarred nylon net with 20 mm bar length was lashed onto the pipe. To ensure the net panels remained submerged in the tanks and in a vertical position the bottom sections of the pipes were filled with sand. The net panels were left to foul for 2 weeks (before being collected and fed to seahorses) at the Atlantic salmon and Ocean trout sea cage farm operated by Van Dieman Aquaculture, Rowella, Tasmania. Panels were hung from a walkway at a depth of 2.5 to 3.5 meters.

To ensure the crustaceans were fresh, net panels were collected each morning and transported to the Aquaculture Centre. The nets used from the previous day were also replaced at this time to ensure that the panels were left to foul for 2 weeks. Due to the difficulty in removing the biofouling from the nets alive the nets were simply placed into the seahorse tanks. The crustaceans, which fell off during

transport were collected and evenly distributed between the replicate tanks. To ensure even distribution over time the tank sequence was changed at every meal.

It was not possible to quantify the amount of biofouling added to each tank every day. *Artemia* was added in excess twice a day to ensure the same feeding strategy in both treatments. The approximate *Artemia* food rate was 10% body weight day^{-1} and food was always present as seahorses were observed to feed all day.

Effect of biofouling crustaceans on the growth and condition of seahorses

The individual wet weight of seahorses in all treatments was measured in week 0, 2, 4, 6 and 8. The total length (back of crown to end of tail) of individual seahorses was measured in week 0 and 8 (initial and final sample times) of the feed trial.

Specific growth rate of seahorses in each treatment was calculated using the equation:

$$SGR = 100.(\ln W_f - \ln W_i)/ \Delta_t$$

Where SGR is specific growth rate (% d^{-1}), W_f is the final weight (g) of seahorses over the time interval that weight was measured, W_i is the initial weight (g) of the seahorses, and Δ_t is the time (days) over which weight was measured.

In the biofouling crustacean diet trial no seahorses died.

Statistical analysis

All statistical analyses were carried out using the SPSS (version 11) software package. The statistical methods described examined the effect of biofouling crustaceans and *Artemia* on the growth and condition of pot-bellied seahorses.

The effect of different treatments on the weight of seahorses and the condition index of seahorses was determined as described in section 2.2.1.

3.2.2. Experiment two. Predator prey relationship and preferred prey type and size of seahorses

The aim of experiment two was to assess the relationship between seahorse length, weight, gape, snout length and eye diameter; assess the relationship between predator and prey size and determine the preferred prey type and size of different aged pot-bellied seahorses by studying gut content analysis data. The data on preferred prey sizes was used to determine the size of the diet and fish in the weaning trials.

This experiment was conducted in the Aquaculture Centre. Seahorses used in this experiment were either transferred from Seahorse World Pty. Ltd. or bred at the Centre. Twenty seahorses of each age class (0, 21, 49, 91, 147, 175 and 203 day old) were kept in individual 20 L tanks within a recirculation system as described in section 2.2.1.

Biofouling crustaceans were collected and fed out as per section 3.2.1. The different aged seahorses (except newborns) were allowed to feed on biofouling crustaceans for 2 weeks before gut content analysis. The newborns were fed on biofouling for 5 days. Gut analysis was undertaken at one sampling time. The age of fish, expressed in relation to the results, refers to the age at sampling (ie after feed period).

The tanks were siphoned daily and seahorses were kept under a photoperiod of 12:12 L:D (0800 - 2000 hours light) with a light intensity of 1.2 - 2.1 $umol.m^{-2}.s^{-1}$ (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters during the trial were: dissolved oxygen (7.0 - 7.5 mg L^{-1}), total ammonia (0 - 0.1 ppm), pH (7.5 - 8.4) and temperature (18 ± 0.5°C).

Gut Content Analysis

A problem with gut content analysis is that prey can be unidentifiable due to digestive processes. To determine the time when seahorses had consumed a regular meal and the prey were not past identification, a baseline study was carried out. The feed times examined were 30 min, 1 h, 2 h, 3 h, 4h, 5h, 6h, 7 h, and 8 h. For the purpose of the experiment, five hours of feeding was decided as the optimum as the prey species were still readily identifiable and the gut of seahorses was full.

Ten seahorses per tank (per age) were used for gut content analysis. Seahorses were euthanased in benzocaine and placed on ice. Prior to dissection the seahorses were weighed and morphological measurements (total length, head length, snout length, eye diameter and gape height) were taken (Figure 3.1). These morphological measurements were made using Sigma Scan Pro 5 (a measurement analysis package) on digital images of the seahorses head, which were taken with a (DC300F Leica) camera on an Olympus (BH-2) dissecting microscope and an IM50 software package. Upon dissection all feed was removed from the gut. The feed items were identified to the lowest possible taxon and the total number of each taxa per gut was recorded. The length and width measurements of each prey item were taken off digital images as per seahorse morphological measurements.

To determine crustaceans available from the net panels and their size biofouling crustaceans were collected every month over a period of a year to allow biofouling organisms present and their change in density to be determined. There were 3 replicate nets for each month and they were stored in 70% alcohol for later analysis. Density was determined for those species consumed by seahorses.

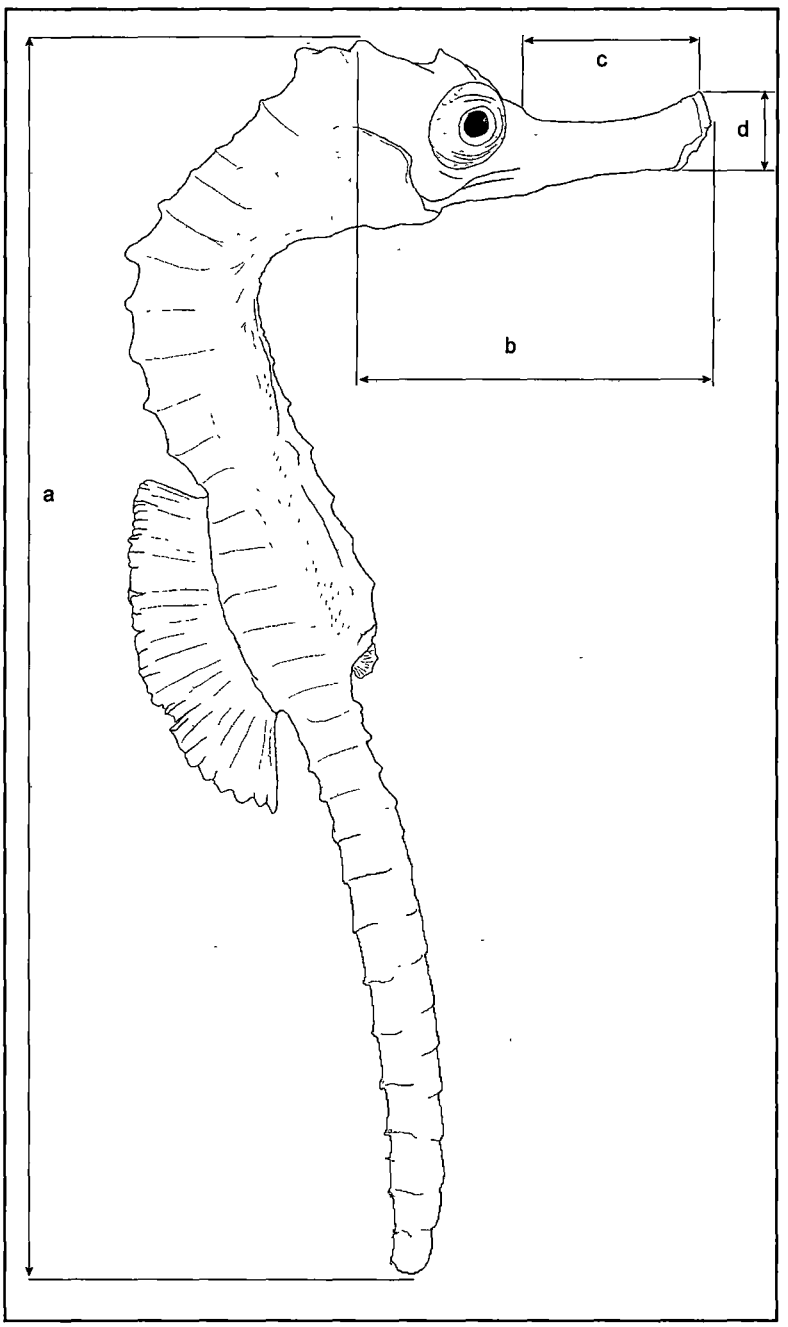


Figure 3.1. The morphological measurements (a) total length, (b) head length, (c) snout length, (d) gape height and (e) eye diameter.

Density was determined by removing all crustaceans from the net and then spreading them out evenly on a tray which had been previously divided up into 30 squares. Crustaceans were counted in 9 randomly selected squares which equates to 30% of the crustaceans present. There were three net panels for each month.

Prey preference selection

Prey preferences of fish may be analysed using prey selection indices. There are a number of selection indices that are appropriate for answering different ecological questions and in the present study the index of Cheeson (1978, 1983) was used based on the constant preference coefficient. Cheeson's index (α_a) may be expressed by the following formula:

$$\alpha_a = r_a P_a^{-1}[\Sigma_{i=1}^{2} r_i p_i^{-1}]^{-1} = e a_d (d a_e)^{-1}[e d^{-1}(a_d a_e^{-1} + b_d b_e^{-1})]^{-1}$$

where a_d is the number of prey animals of species a in the predators diet, b_d is the number of all other prey animals in the diet, a_e is the number of prey animals of species a in the environment, b_e is the number of all other prey animals in the environment, d is the total number of all animals in the diet, e is the total number of all animals in the environment, r_a is the proportion of prey species a in the diet and P_a is the proportion in the environment (Pinnegar et al., 2003).

Cheeson's index (α_a) (also known as the 'standardised forage ratios') has become popular because of its use in food-web modelling packages (Christensen et al., 2000). The standardised forage ratio as presented, ranges between 0 and 1, with $\alpha_a = 0$ representing complete avoidance and $\alpha_a = 1$ exclusive feeding on a particular prey type. The index is independent of prey availability and as α_a was calculated for a 'portfolio' of four species an α_a equal to 0.25 denotes random feeding (ie a particular prey is taken by the predator in exactly the same proportions as in the environment) and an $\alpha_a >$ than 0.25 represents positive selection.

Statistical analysis

Statistical analyses were carried out using the SPSS (version 11) software package. The statistical methods described morphological relationships and predator prey relations.

The morphological changes associated with age were described by way of principal component analysis (PCA), a multivariate ordination technique that enables observations in multidimensional space to be examined in fewer dimensions and identifies and summarises major patterns of variation. The new axes are combinations of the original variables so the relationship between the variables and new axes is also described and the axes are orthogonal to one another making the variation explained by each axis unique. The analysis calculates the percentage of variation in the data set explained by each axis and for the purpose of this study a covariance matrix of $\log_{10}$ transformed data was used as this is the usual form to examine allometric relationships in multivariate morphometric data and allows relationships between the variables and how they covary to be described (Jolicouer, 1963).

Predator-prey size relations were tested by stepwise linear regression between mean prey width and predator size (total length, head length, snout length, gape and eye diameter) (Zar, 1996). Prey width was used as it is known to be superior to prey length for defining size relation in trophic dynamic analysis and is the accepted measure that limits the size of prey ingested by fish (Fernandez-Diaz et al., 1994; Ghan & Sprules, 1993; Govoni, 1986; Morato et al., 2000; Pearre, 1986).

To determine whether different aged seahorses consumed different sized prey a one way analysis of variance (ANOVA) using the General Linear Model was used and Tukey's Honestly Significant Difference (HSD) test was used to

determine significance between means. The level of significance of $p < 0.05$ was used.

3.2.3. Experiment three. Effect of copepods on the growth and condition of seahorses

Seahorses were fed copepods to assess whether early juvenile pot-bellied seahorses would readily consume copepods and to determine whether fish growth and condition was similar or better than seahorses fed enriched *Artemia*.

This experiment was conducted in the Aquaculture Centre. A total of 160 three-week old pot-bellied seahorses were transferred from Seahorse World Pty. Ltd. Seahorses were treated (Acriflavine™ and 50 ppm formalin) and 20 fish were distributed randomly to each tank. Each tank was randomly allotted a treatment (1 - 2, copepods or *Artemia*) and replicate (1 - 4) number.

This experiment was planned to run for 8 weeks as a standard experimental duration but was ended early (week 6) as the copepod culture started to decline in week 5 of the trial.

System configuration

Pot-bellied seahorses were maintained in eight 20 L tanks within a recirculation system as described in section 2.2.1. and maintained experimentally as described in section 3.2.1.

The seahorses were kept under a photoperiod of 12:12 L:D (0800 - 2000 hours light) with a light intensity of 1.3 - 2.2 $umol.m^{-2}.s^{-1}$ (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters during the trial were: salinity (32 ppt ± 2); temperature (18 ± 0.5°C); total ammonia (0 - 0.25 mg/L); nitrite (0 - 0.25 mg/L); nitrate (5 - 10 mg/L); pH (7.8 - 8).

Feed

Artemia were hatched as per section 2.2.1. and were enriched with Algamac 3050™ for 15 hours (for daily meal 1) and then re-enriched for a further 4 hours (daily meal 2) hours at a dose rate of 0.2 g L^{-1}.

Harpacticoid copepods (*Tisbe* sp.) were grown at the Aquaculture Centre in a 400 L tank in the 28°C temperature control room. They were fed a combination of the microalgae *Tetraselmis suecia*, *Isochrysis galbana*, and *Pavlova lutheri* and this was supplemented with baker's yeast. As *Tisbe* sp. is a surface dwelling species, sheets of 60 um nylon mesh and pot scourers were placed in the tank to provide substrate. Copepods and copepodids were harvested off the substrates at each feed time. To ensure a breeding population was maintained adults were screened and returned to the tank and only early stage copepodids were distributed to the fish.

Seahorses were fed at a feed rate (dry feed) of 5% body weight day^{-1} (based on results from Chapter two) over a morning (0900 h) and an afternoon (1300 h) meal. To ensure the distribution of *Artemia* and copepods was even among the replicates (four) in each treatment the tank sequence was changed at each feed time. Copepods were quantified by using their dry weight to determine the number of copepods required per feed and counting sub-samples under a microscope.

Effect of copepods on the growth of seahorses

The individual weight of seahorses in all treatments was measured in week 0, 2, 4 and 6. The total length (back of crown to end of tail) of individual seahorses was measured in week 0 and 6 (initial and final sample times) of the feed trial.

Specific growth rate of seahorses in each treatment was calculated as per section 3.2.2.

Mortalities were removed from the tank when observed and recorded daily. In this experiment 6.9% of the seahorses died (6 seahorses in the copepod treatment and 4 in the *Artemia* treatment). Dead fish were not replaced in this trial.

Statistical analysis

All statistical analyses were carried out using the SPSS (version 11) software package. The statistical methods examined the effect of copepods and *Artemia* as diets on the growth and condition of pot-bellied seahorses.

The effect of different treatments on the weight of seahorses and the condition index of seahorses was determined as described in section 2.2.1.

3.2.4. Experiment four. Effect of frozen diets on the growth and condition of seahorses and the development of a weaning protocol

Seahorses were fed frozen amphipods and frozen mysids to determine (i) whether amphipods or mysids promoted better growth and condition in seahorses, (ii) whether the growth of seahorses fed frozen diets was similar or better than those seahorses fed live enriched instar II *Artemia* and (iii) the optimal weaning protocol for seahorses.

This experiment was conducted in the Aquaculture Centre. A total of 740 (320 for each of the 2 experiments - amphipods and mysids) thirteen-week old seahorses (based on size of mysids available and findings from section 3.2.3) were transferred from Seahorse World Pty. Ltd. Seahorses were treated with Acriflavine™ and 50 ppm formalin for 24 hours to remove skin protozoans and 20 fish were distributed randomly to each tank (16 tanks). Each tank was randomly allotted a treatment (1 - 4, *Artemia* only, no weaning, 10 day weaning or 16 day weaning) and replicate number (1 - 4).

System configuration

Pot-bellied seahorses for each frozen diet experiment were maintained in sixteen 20 L tanks within a recirculation system as described in section 2.2.1. and maintained experimentally as described in section 3.2.1. The flow rate was maintained at 2.5 L min^{-1}.

The seahorses were kept under a photoperiod of 12:12 L:D (0800 - 2000 hours light) with a light intensity of 1.3 - 2.2 $umol.m^{-2}.s^{-1}$ (Li-Cor Model Li-250 Light Meter) at the water surface of the tanks. The range of water quality parameters during the trial were: salinity (32 ppt ± 2); temperature (18 ± 0.5°C); total ammonia (0 - 0.25 mg/L); nitrite (0 - 0.25 mg/L), nitrate (5 - 10 mg/L); and pH (7.8 - 8).

Feed and weaning protocol

Artemia were hatched as per section 2.2.1. and were enriched with Algamac 3050™ for 15 hours (daily meal 1) and re-enriched for a further 4 and 9 hours (daily meal 2 and 3). Frozen mysids *(Paramesodopsis rufa, Tenagomysis* sp) used were bought by the University from Mike Scott. The amphipods (beach hopper sp) were collected from a beach north of Granville Harbour, Tasmania and upon arriving at the Aquaculture Centre they were sorted into different sizes by sieving them through a number of different sized screens and subsequently frozen in small batches. Frozen mysids and amphipods were thawed and rinsed in freshwater prior to being distributed to the fish.

The weaning protocols examined were (i) *Artemia* only, (ii) no weaning period, (iii) a 10 day weaning period and (iv) a 16 day weaning period (Figure 3.2, 3.3, 3.4). For the treatments with a changeover period the amount of *Artemia* was reduced by 25% after allowing the seahorses to feed on the mixed ration for 3 and 5 days for the 10 day and 16 day weaning periods respectively. The frozen diet

ration was fed to the fish in the morning and *Artemia* were fed in the afternoon; the new diet being fed when fish show the highest appetite.

Seahorses were fed twice a day (0800 h and 1300 h) at a total feed rate of 5% body weight day^{-1}. The amount of each feed was calculated and feed was counted out for each meal for every treatment for the first 28 days of the two month trial and the amount of food consumed (ie amount of feed offered less food removed from tank) was recorded daily for both the morning and afternoon meals. Feed was recalculated fortnightly after each weight check.

The amount of *Artemia* to feed was calculated on the basis of the weight of cysts (section 2.2.1). The amount of amphipods and mysids to feed was also calculated based on the weight of the animals and the required feed rate. Mean individual amphipod and mysid weights were 0.59 mg and 3.0 mg (dry weight) respectively.

By way of example, if a seahorse weighed 0.3 g and seahorses were fed at 5% body weight day^{-1} then they would require 0.015 g of food per day. If seahorses require 0.015 g and are fed amphipods which weigh 0.00059 g each then 25 amphipods (0.015/0.00059) will be required for each seahorse (Appendix 9.7).

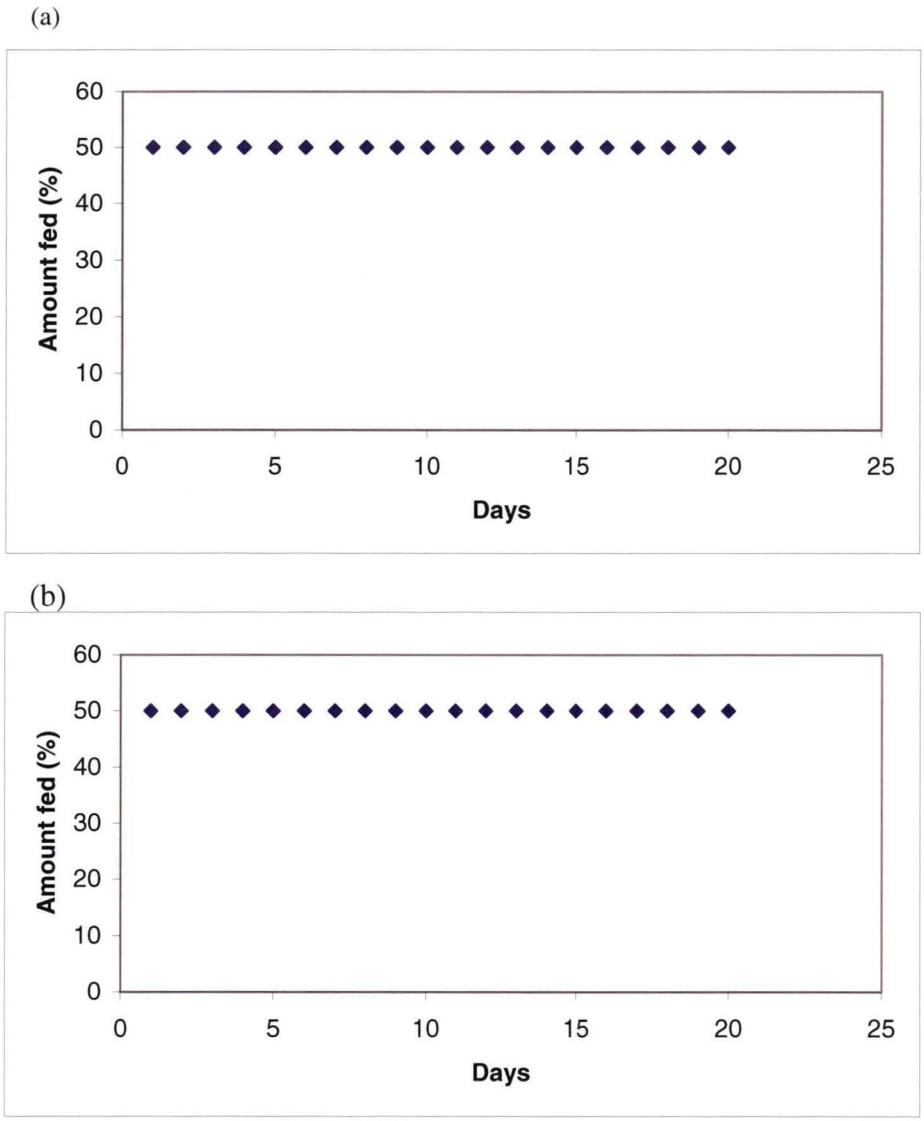
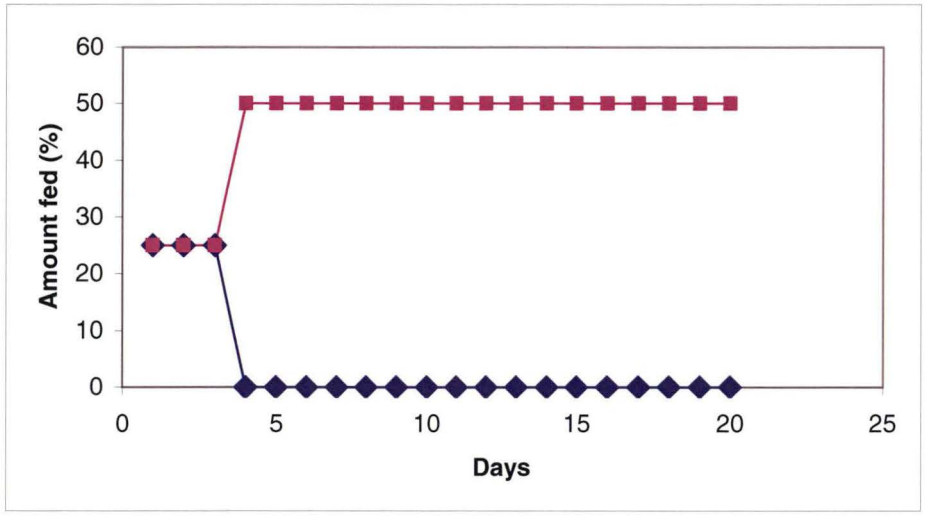


Figure 3.2. The (a) morning and (b) afternoon weaning protocols for the *Artemia* only and no weaning period treatments. ♦*Artemia* or frozen diet.

(a)



(b)

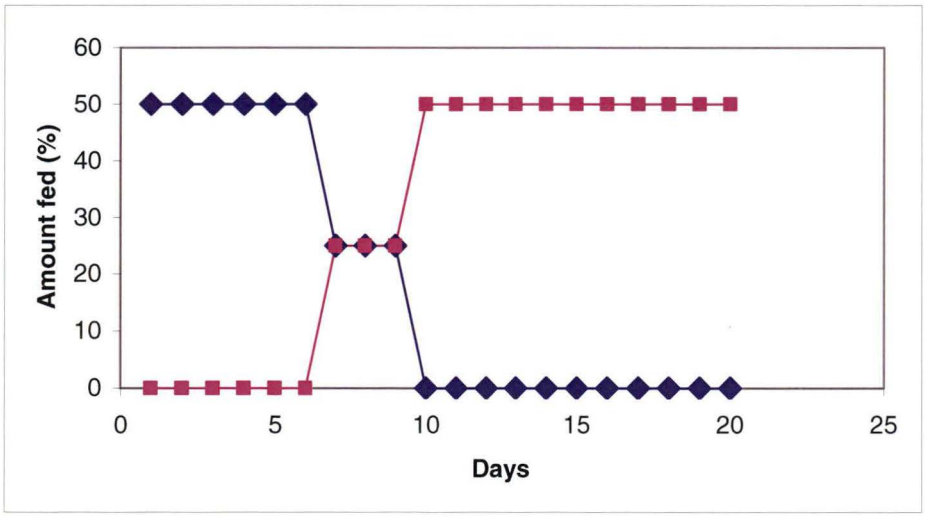
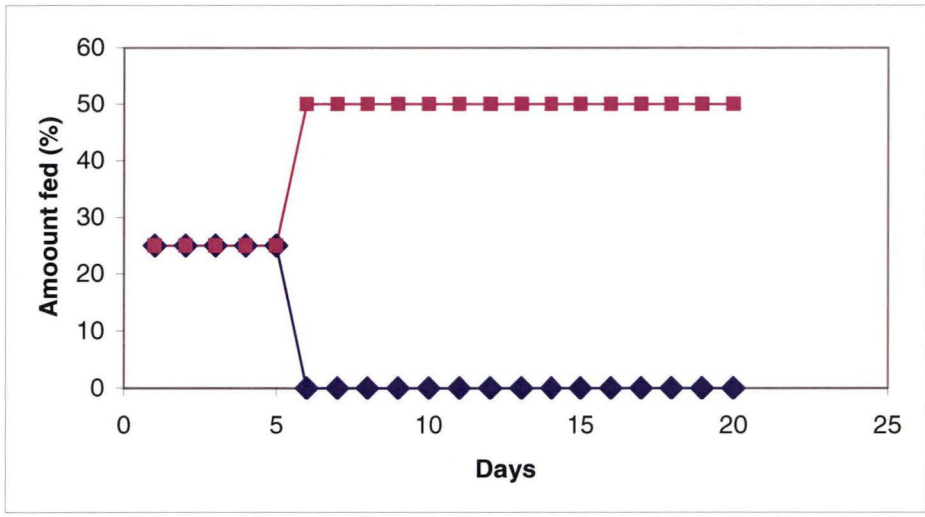


Figure 3.3. The (a) morning and (b) afternoon weaning protocol for the 10 day weaning period treatment. ♦*Artemia*, ■ frozen diet.

(a)



(b)

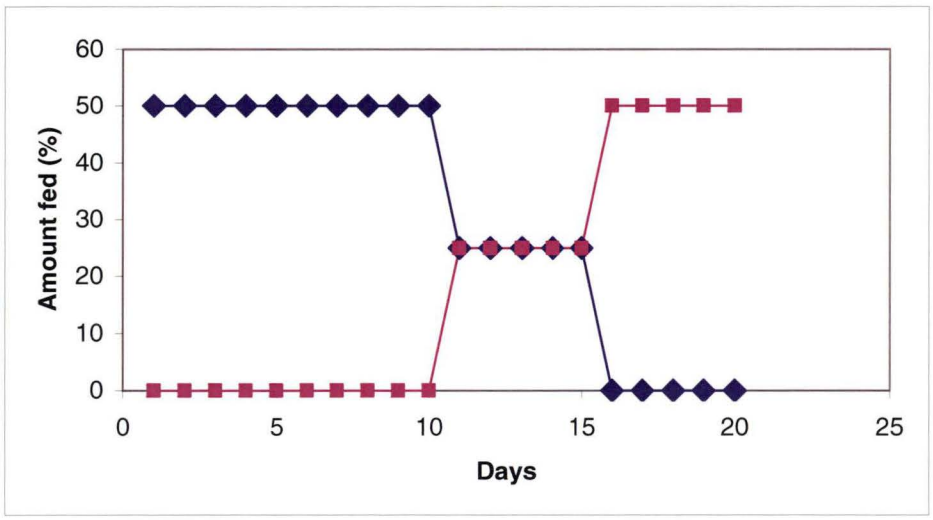


Figure 3.4. The (a) morning and (b) afternoon weaning protocol for the 16 day weaning period treatment. ♦*Artemia*, ■ frozen diet.

Effect of frozen diets on the growth of seahorses

The individual weight of seahorses in all treatments was measured in week 0, 2, 4, 6 and 8. The total length (back of crown to end of tail) of individual seahorses was measured in week 0 and week 8 (initial and final sample times) of the feed trial.

Specific growth rate of seahorses in each treatment was calculated as per section 3.2.2.

Food conversion ratio was calculated as

FCR = amount of food consumed over time/ weight change over time

= (number of prey added - uneaten food) / (final - initial fish weight)

Feed efficiency was calculated as

$$FE = (BW_f - BW_i) / \Sigma FW_t$$

Where BW_f is final body weight, BW_i is initial body weight and ΣFW_t is the amount of food consumed over the trial.

Mortalities were removed as observed and recorded daily. In this trial a total of 6.9% of the seahorses died (with 4 in the no weaning treatment and 6 in the 10 day weaning period). Mortalities were not replaced in this trial.

Statistical analysis

Statistical analysis was carried out using the SPSS (version 11) software package. The statistical methods described the effect of frozen diets and different weaning protocols on the growth and condition of the seahorses.

The effect of different treatments on the weight of seahorses and the condition index of seahorses was determined as described in section 2.2.1.

3.3. RESULTS

3.3.1. EXPERIMENT ONE. EFFECT OF BIOFOULING CRUSTACEANS ON THE GROWTH AND CONDITION OF SEAHORSES

Growth of seahorses

There was no significant difference ($F = 0.005$, df 1, $p > 0.05$) between the weight of pot-bellied seahorses in the *Artemia* and biofouling treatments at the beginning of the growth trial (Figure 3.5, Appendix 9.8). At the completion of the trial there was no significant difference between the weight of seahorses fed *Artemia* enriched with Algamac 3050™ and those which foraged on the biofouling plates ($F = 0.982$, df 1, $p > 0.05$) (Figure 3.5, Appendix 9.8).

Specific growth rates of seahorses fed *Artemia* enriched with Algamac 3050™ and biofouling from the beginning to the end of the feed trial were 1.2402% d^{-1} and 1.4618% d^{-1} respectively.

Condition indices of seahorses in the dietary feed trial were determined by examining length to weight relationships. A significant difference ($F = 7.407$, df 1, $p < 0.05$) was found between the condition index of seahorses in different dietary treatments; seahorses fed *Artemia* had a lower slope (1.196) thus poorer condition then the seahorses fed biofouling (1.804) (Table 3.1). There was little to no variation between the weights and lengths of seahorses in the *Artemia* and biofouling treatments at both the beginning and on completion of the trial (Table 3.1).

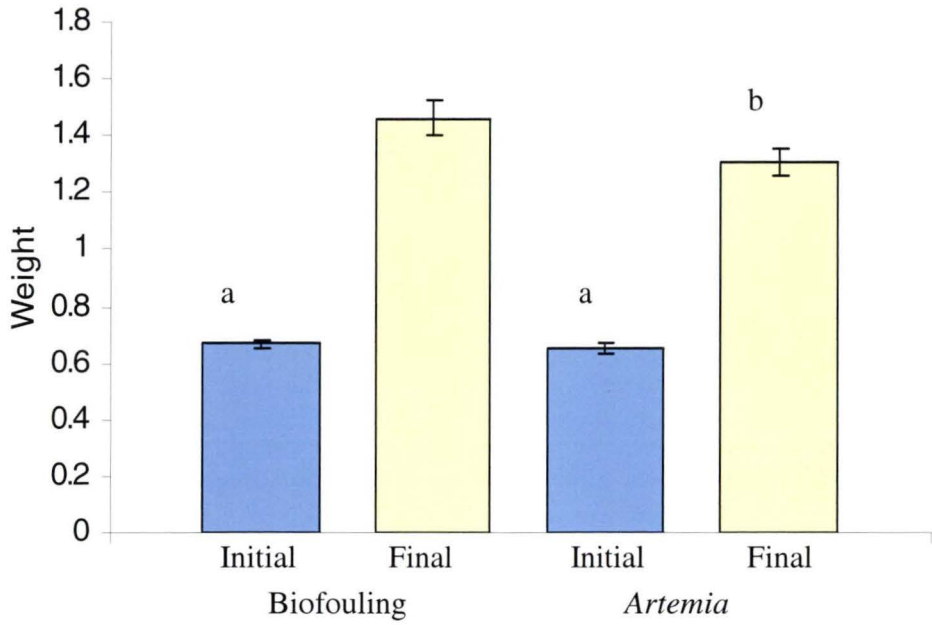


Figure 3.5. The initial ♦ and final ♦ mean (± S.E) weight of pot-bellied seahorses fed biofouling crustaceans and *Artemia* enriched with Algamac 3050™ in the alternative feed trial. Means which did not significantly differ in Tukey's HSD test share the same common superscript. (Refer to Appendix 9.8 for further information on initial and final mean weights).

Table 3.1. Effect of diet (biofouling crustaceans and *Artemia* enriched with Algamac 3050™) on the length to weight relationship (condition index) of the pot-bellied seahorses and the initial and final coefficient of variation for weight and length for each diet treatment (a = elevation of the line; b = slope of the line, lower slope = poorer condition; r^2 = explanatory variable).

	Condition Index				Coefficient of Variation			
					Weight		Length	
Treatment	a	b	r^2	p	Initial	Final	Initial	Final
Artemia	-1.95	1.196	.595	<0.05	0.1872	0.2139	0.0592	0.0811
Biofouling	-3.10	1.804	.789	<0.05	0.1672	0.2692	0.0458	0.0880

(Regression equation: $\log_{(\chi+1)}$ weight = b $\log_{(\chi+1)}$ length + $\log_{(\chi+1)}$ a)

3.3.2. Experiment Two. Relationship between seahorses and their prey

Seahorse morphology

The length of the head, length of the snout, gape height and eye diameter of seahorses were found to increase with seahorse length and weight (Table 3.2). When a stepwise regression was run to determine the best predictor for prey width (seahorse trait used to determine optimal prey size) it was found there was a significant relationship between seahorse total length and prey width, gape height and prey width, and snout length and prey width (Table 3.3). As snout and gape measurements are more difficult and time consuming to take, seahorse length is the best practical predictor of optimal prey sizes for different aged seahorses.

The biggest source of variation in the data was the size of the seahorses (Figure 3.6). The first principal component axis described 95.1% of the variation among individuals, which means that all variables (total length of seahorse, head length, snout length, gape height, eye diameter) were increasing in relative size (Table 3.4). The coefficients for each variable in the first eigenvector describe the relative growth rates of all the components simultaneously (Shea, 1985). The shape of newborn, 21, and 49 day old seahorses changed rapidly before relative growth started to slow in 91 day old seahorses. Thus seahorses attained their final body shape at approximately 50 mm in length (91 days old) (Figure 3.7) and most of the variation expressed along the axis was due to growth of smaller individuals (seahorse length < 50 mm).

Table 3.2. The mean (± SE) total length, weight, head length, snout length, gape (height) and eye diameter of different aged pot-bellied seahorses. (Seahorses in this experiment were from mixed cohorts. Some were bred in the Key Centre and others were collected from Seahorse World Pty. Ltd.)

Seahorse age (days)	seahorse length (mm ± SE)	seahorse weight (g ± SE)	head length (mm ± SE)	snout length (mm ± SE)	gape (mm ± SE)	eye diameter (mm ± SE)
newborns	15.66 ± 0.494	0.0078± 0.0005	3.84 ± 0.137	1.81 ± 0.064	1.24 ± 0.035	0.97 ± 0.031
21	25.27 ± 0.497	0.024 ± 0.0011	6.31 ± 0.082	3.07 ± 0.042	1.68 ± 0.026	1.43 ± 0.024
49	36.56 ± 0.606	0.078 ± 0.0025	9.35 ± 0.091	4.72 ± 0.070	2.15 ± 0.068	2.08 ± 0.050
91	50.22 ± 0.937	0.212 ± 0.0158	11.81 ± 0.217	5.54 ± 0.182	2.77 ± 0.055	2.72 ± 0.068
147	71.11 ± 2.293	0.844 ± 0.0567	13.68 ± 0.049	4.86 ± 0.030	3.65 ± 0.045	3.39 ± 0.094
175	79.44 ± 2.635	0.993 ± 0.0807	14.29 ± 0.133	5.71 ± 0.197	3.91 ± 0.105	3.46 ± 0.130
203	84.66 ± 2.756	1.214 ± 0.1329	14.96 ± 0.303	6.59 ± 0.203	4.25 ± 0.181	3.49 ± 0.094

Table 3.3. Relationship between prey width and three morphological traits of seahorses, total length, snout length and gape height. All measures are $\log_{10}$ transformed.

	a	b	Adjusted r^2	df	F	Sig.
Seahorse length	-1.366	0.770	0.604	547	837.322	$p < 0.05$
Snout length	-1.406	-0.206	0.607	546	424.223	$p < 0.05$
Gape height	-1.204	0.388	0.609	545	285.950	$p < 0.05$

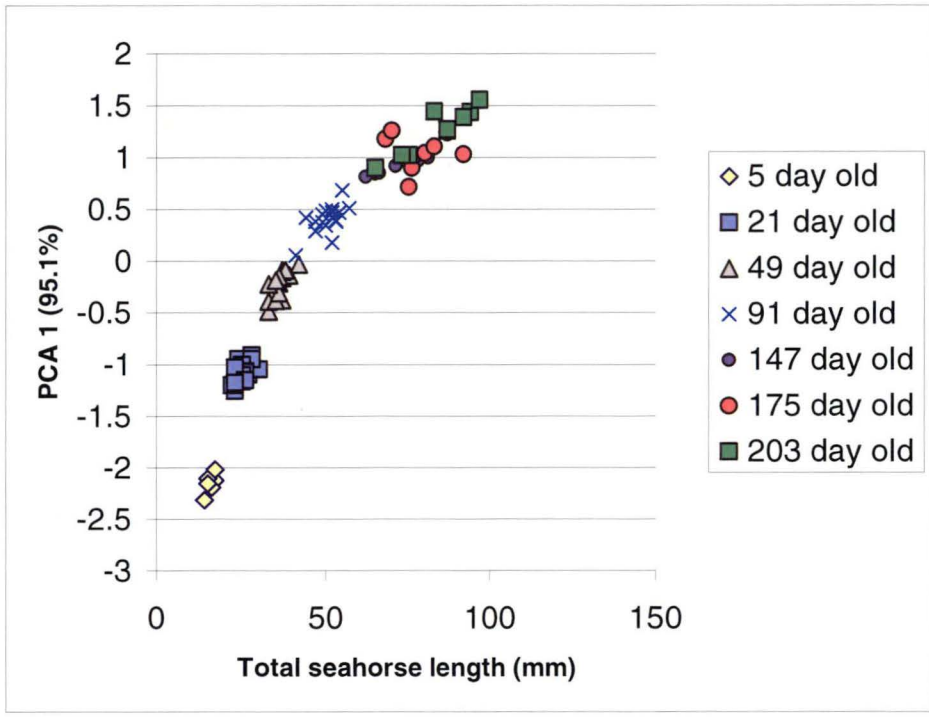


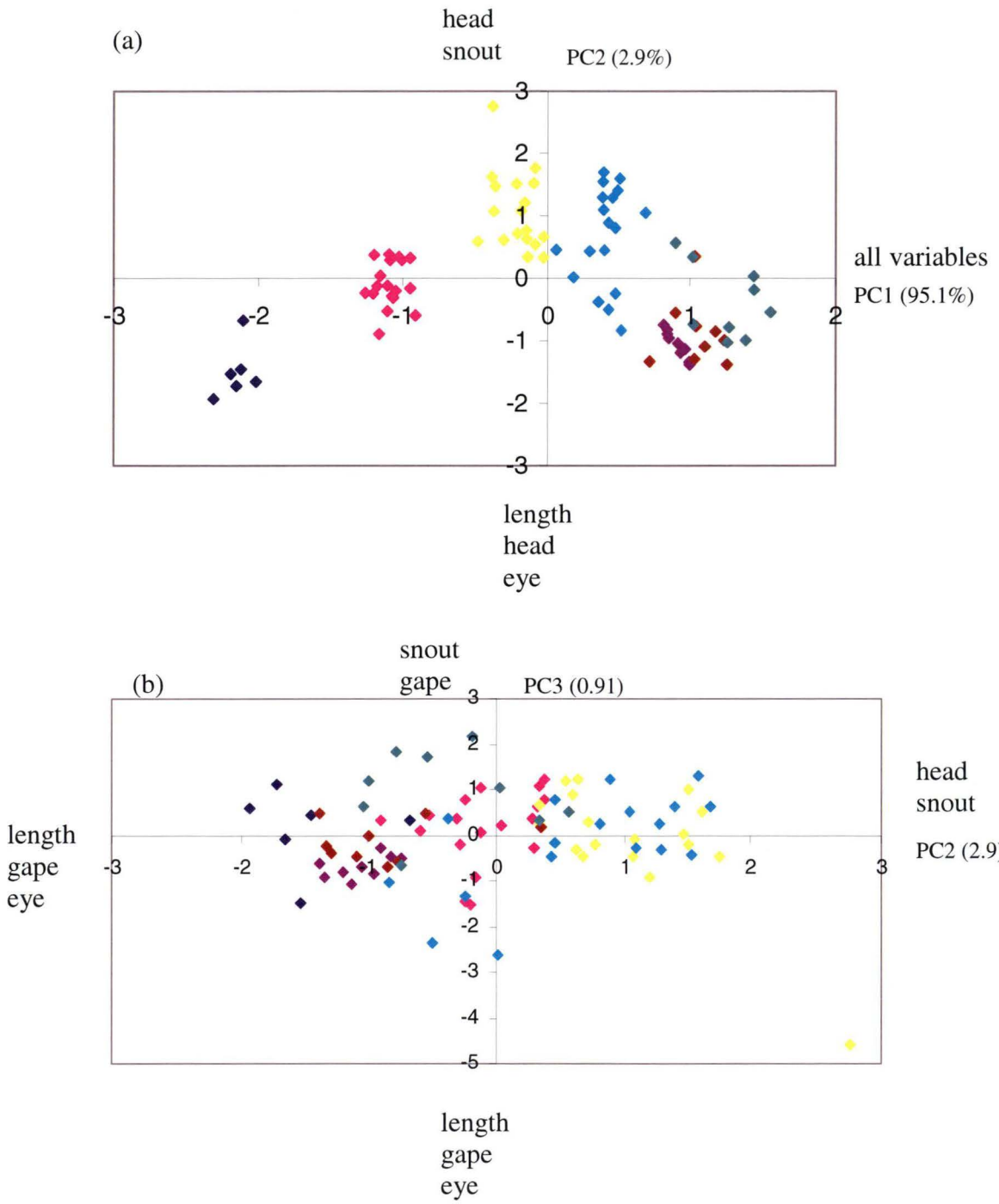
Figure 3.6. Relative growth of seahorse body structures (head length, snout length, gape height and eye diameter). The relationship between the principal component score of each individual seahorse against total seahorse length.

Table 3.4. Relative growth of seahorse body structures (total length, head length, snout length, gape height and eye diameter). The coefficients for the first three components of the analysis on length variables. Data are $\log_{10}$ transformed and the covariance matrix was used.

Variable	Eigenvector		
	PCA1 (95.1%)	PCA2 (2.9%)	PCA3 (0.9%)
Seahorse length	0.308	-1.062	-1.359
Head length	0.191	0.650	-0.979
Snout length	0.145	1.779	1.404
Gape height	0.180	-1.104	3.249
Eye diameter	0.200	-0.184	-2.177

Seahorse length grew proportionally faster than head length, snout length, gape height and eye diameter; head length, gape height and eye diameter grew at similar relative growth rates and snout length grew at a slower rate (Table 3.4). It was possible to describe gross changes in the shape of different aged seahorses on the first axis. There was some additional variation described on the second and third axes as it was found that head and snout length were proportionally larger in 49 and 91 day old seahorses and gape is proportionally larger in 21, 49 and 91 day old seahorses (Figure 3.7ab, Table 3.4).

Figure 3.7. Principal component analysis plots (a) PCA 1 against PCA 2 and (b) PCA 2 against PCA 3 of the relative growth of body structures of different aged pot-bellied seahorses. Data are $\log_{10}$ transformed. Percentages indicate how much variation in the data set has been described by each principal component axis. ♦ 5 day old, ♦ 21 day old, ♦ 49 day old, ♦ 91 day old, ♦ 147 day old, ♦175 day old and ♦ 203 day old seahorses.


(a)
head
snout
PC2 (2.9%)
all variables
PC1 (95.1%)
length
head
eye
(b)
snout
gape
PC3 (0.91)
length
gape
eye
head
snout
PC2 (2.9)
length
gape
eye

Biofouling composition

The main prey types found on the biofouling nets included copepods, amphipods (*Hippomedon* sp and *Biribus* sp) and caprellids (*Caprella* sp). Other biofouling organisms found on nets included ostracods, other malacostracans such as cumacead shrimp and isopods (Anthuridea and Valifera), polycheate worms, barnacles (Lepadomorpha) and a number of crab species. The number of crustaceans increased from October onwards and then started to decline from January onwards with minimal numbers being present at the end of April (Table 3.5ab). It was also noted that hydroids fouled the nets and became a lot denser from March onwards.

DIET COMPOSITION AND SIZE OF PREY CONSUMED BY DIFFERENT AGED SEAHORSES

The main prey items consumed by seahorses from the biofouling panels were copepods, *Hippomedon* sp, *Biribus* sp and *Caprella* sp. There appeared to be a definite shift in prey selection relative to seahorse age with newborn seahorses consuming mainly copepods and a number of *Hippomedon* sp, 21 and 49 day old seahorses consuming a small number of copepods and both *Hippomedon* sp and *Biribus* sp, and 91, 147, 175 and 203 day old seahorses consuming both amphipod species and caprellids (*Caprella* sp) (Figure 3.8 - 3.14).

A significant difference ($F = 55.141$, df 6, $p < 0.05$) between prey widths for each age category of seahorses tested was found. The range of prey sizes consumed, expanded with increasing seahorse length with newborns, 21, 49, 91, 147, 175 and 203 day old seahorses consuming (out of the possible range of biofouling crustaceans present) prey that ranged between 0.14 - 0.4 mm, 0.17 - 0.51 mm, 0.2 - 0.73 mm, 0.18 - 1.27 mm, 0.18 - 1.35 mm, 0.33 - 1.48 mm, and 0.33 - 1.70 mm respectively (Figure 3.8 - 3.14). It was also noted that the upper (95% quartile) and lower (5% quartile) limits of the prey width consumed by different aged seahorses changed at different rates and that the relationship between seahorse age and maximum prey size was greater than the relationship with minimum prey size

(Figure 3.15) which indicates that the large predators continued to consume small prey while small predators were limited to the smaller prey.

Predator preference

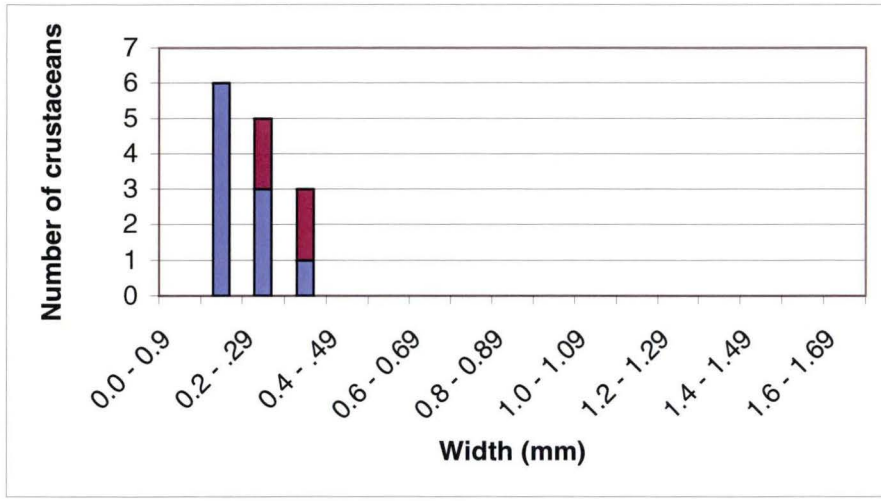
In terms of Cheeson's standardised forage ratio α_a, relative to a portfolio of the 4 key crustacean prey and assuming that anything greater than 0.25 (α_a = k-1) represents positive selection, then newborn, 21 and 49 day old seahorses completely avoided caprellids and 91, 147, 175 and 203 day old seahorses avoided copepods. In addition newborn, 21 and 49 day old seahorses showed a particular preference for copepods with 21 and 49 day old seahorses also positively selecting for *Hippomedon* sp and *Biribus* sp. Larger 147 and 175 day old seahorses positively selected for both *Hippomedon* sp and *Biribus* sp and 203 day old seahorses positively selected for *Biribus* sp and *Caprella* sp (Figure 3.16).

Table 3.5a. The number (mean ± SE) of harpacticoid copepods and amphipods (*Hippomedon* sp., *Biribus* sp and *Caprella* sp.) found on the biofouling nets over late spring and summer 2002/2003.

Species	October	November	December	January	February	March	April
copepods	98 ± 15	223 ± 46	341 ± 78	320 ± 67	89 ± 21	82 ± 15	0
Hippomedon sp	624 ± 47	1016 ± 99	1452 ± 136	1499 ± 158	950 ± 98	927 ± 102	920 ± 37
Biribus sp	301 ± 31	557 ± 52	869 ± 73	768 ± 60	433 ± 54	388 ± 43	293 ± 12
Caprella sp	217 ± 26	550 ± 32	510 ± 64	431 ± 48	239 ± 63	282 ± 35	167 ± 19

Table 3.5b. The number (mean ± SE) of harpacticoid copepods and amphipods (*Hippomedon* sp., *Biribus* sp. and *Caprella* sp.) found on the biofouling nets over late autumn and winter 2003.

Species	May	June	July	August	September
copepods	00 ± 00	00 ± 00	00 ± 00	00 ± 00	32± 04
Hippomedon sp	412 ± 24	00 ± 00	00 ± 00	00 ± 00	207 ± 34
Biribus sp	98 ± 19	00 ± 00	00 ± 00	00 ± 00	83 ± 11
Caprella sp	52 ± 06	00 ± 00	00 ± 00	00 ± 00	72 ± 22



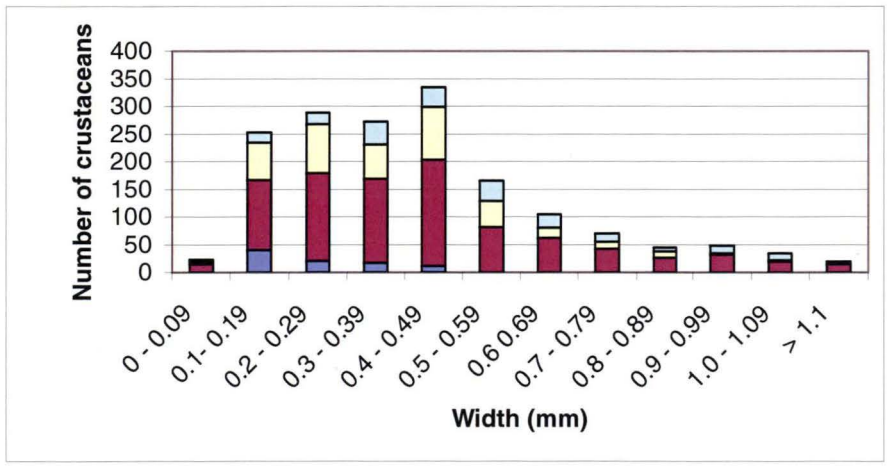
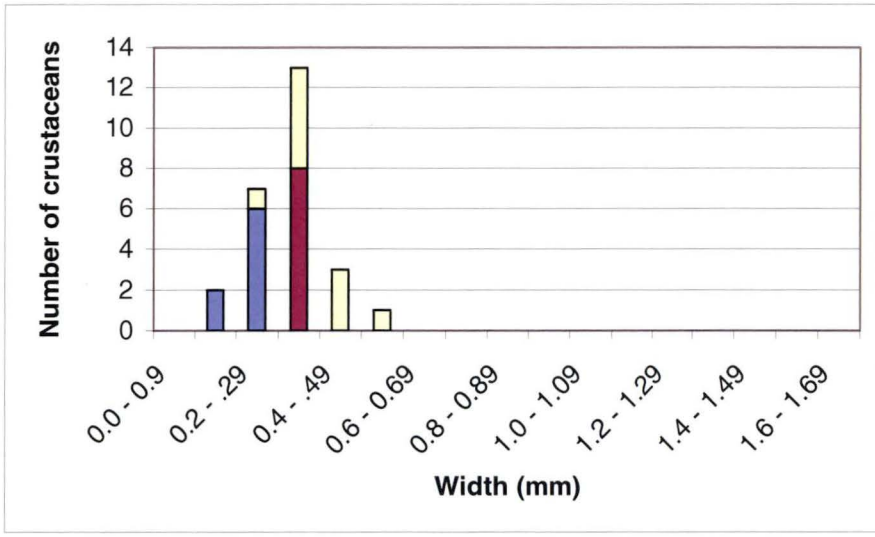


Figure 3.8. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by newborn seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in February when gut content of newborn seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.



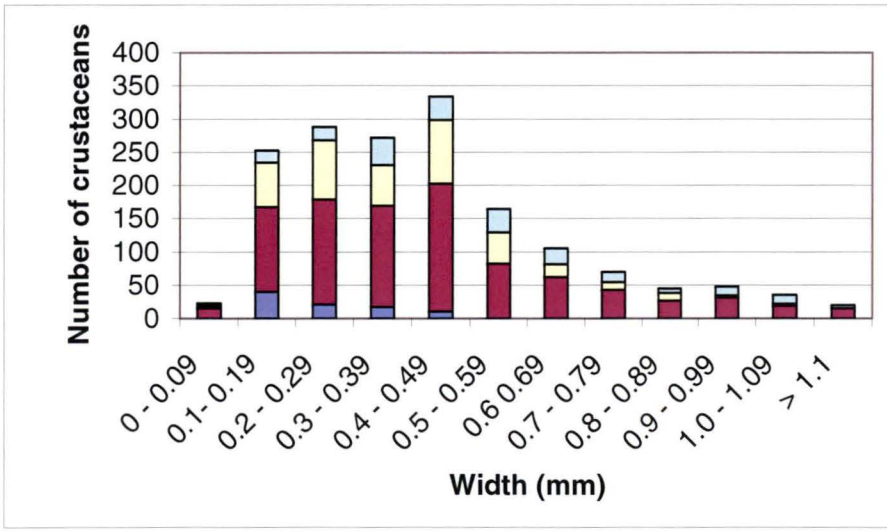
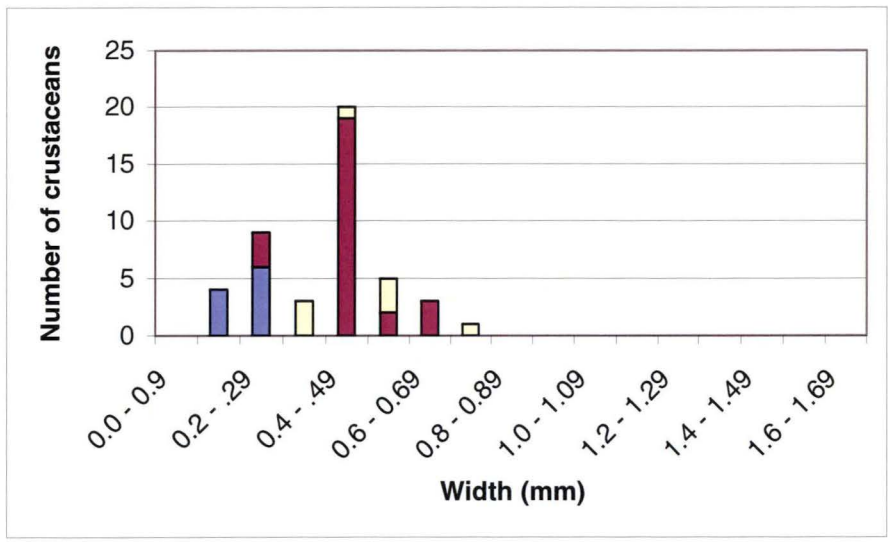


Figure 3.9. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 21 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in February when gut content of 21 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.



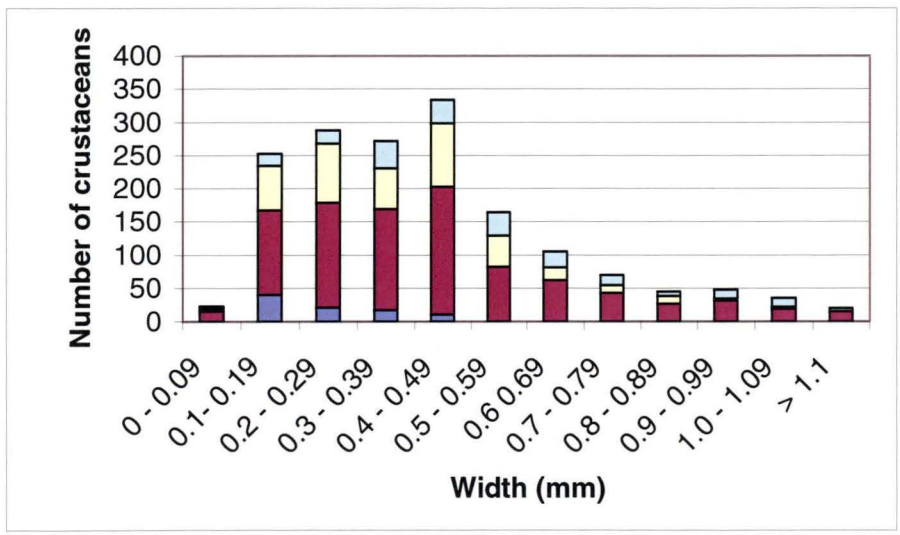
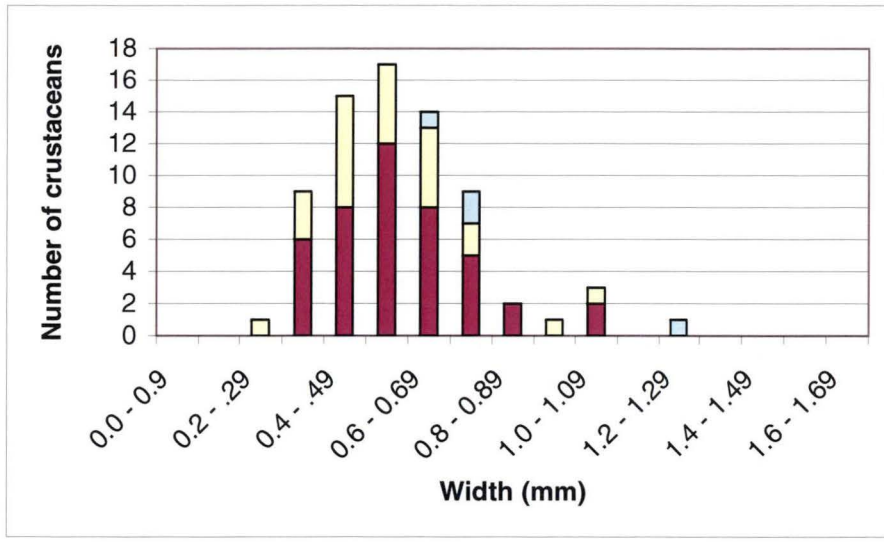


Figure 3.10. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 49 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in February when gut content of 49 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.



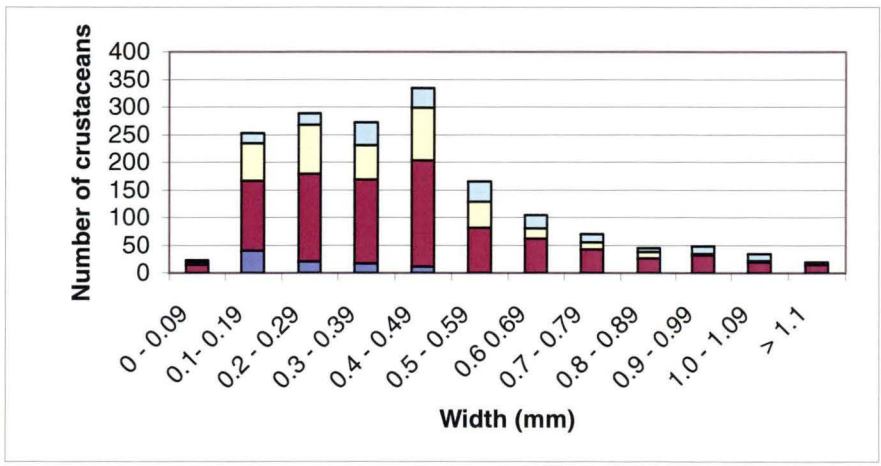
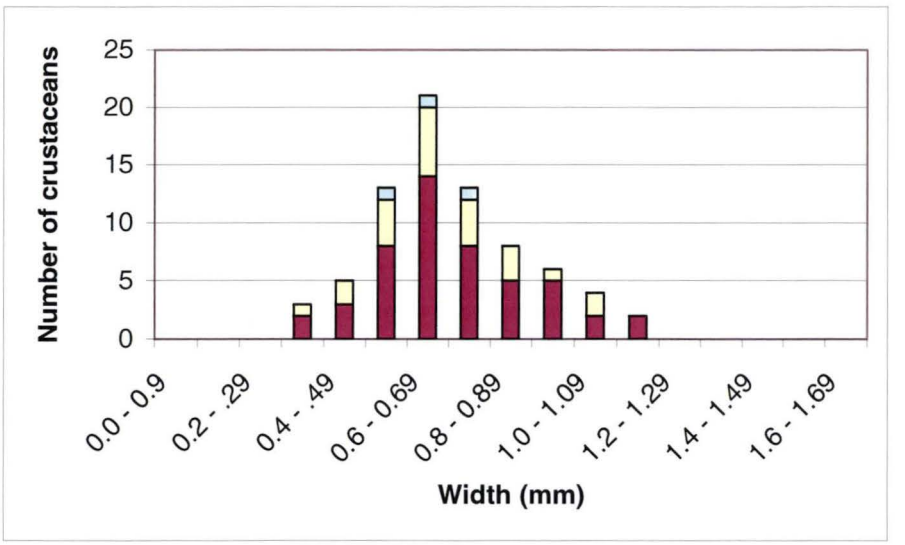


Figure 3.11. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 91 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in February when gut content of 91 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.



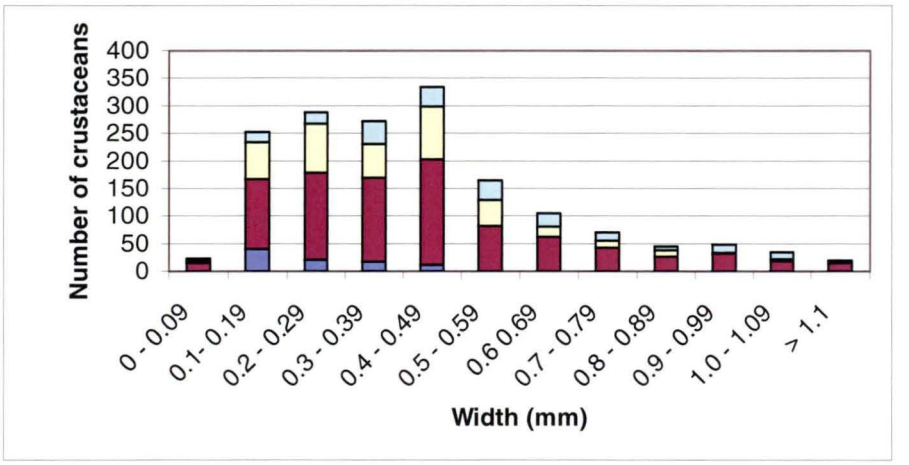


Figure 3.12. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 147 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in February when gut content of 147 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.

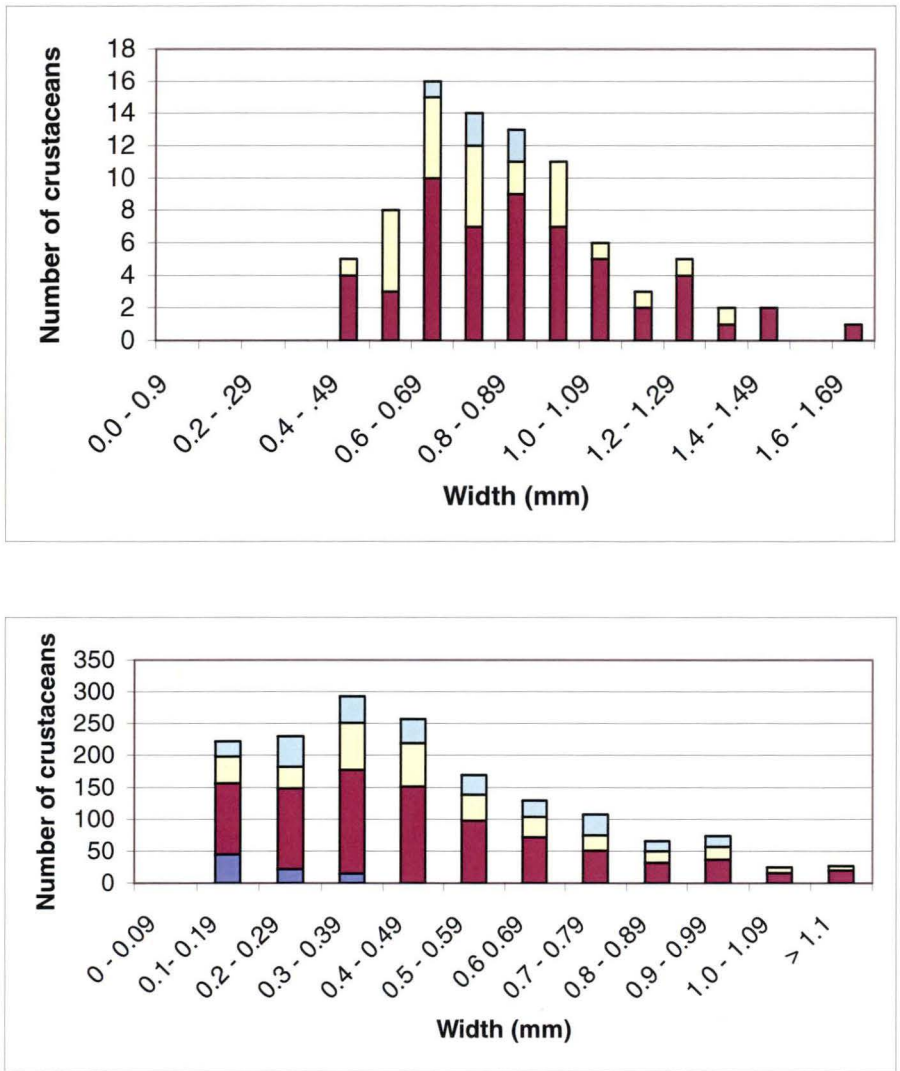
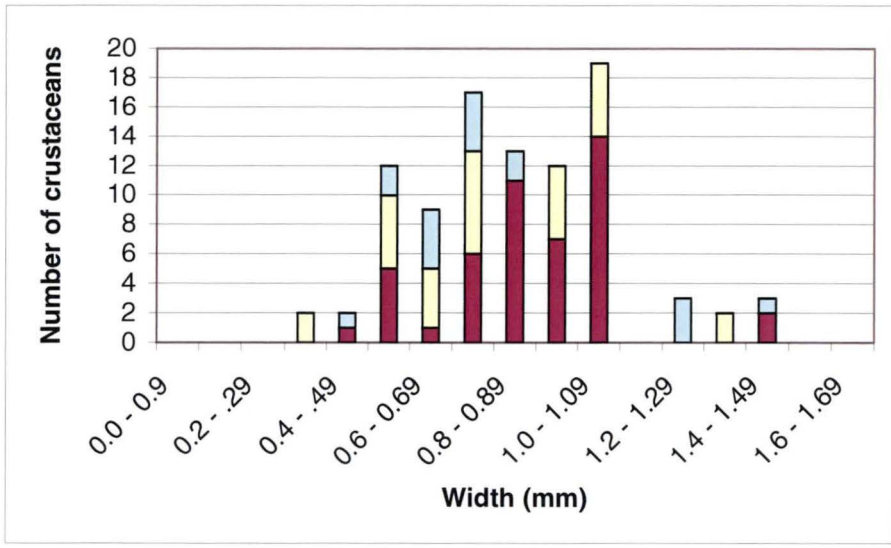


Figure 3.13. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 175 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in March when gut content of 175 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.



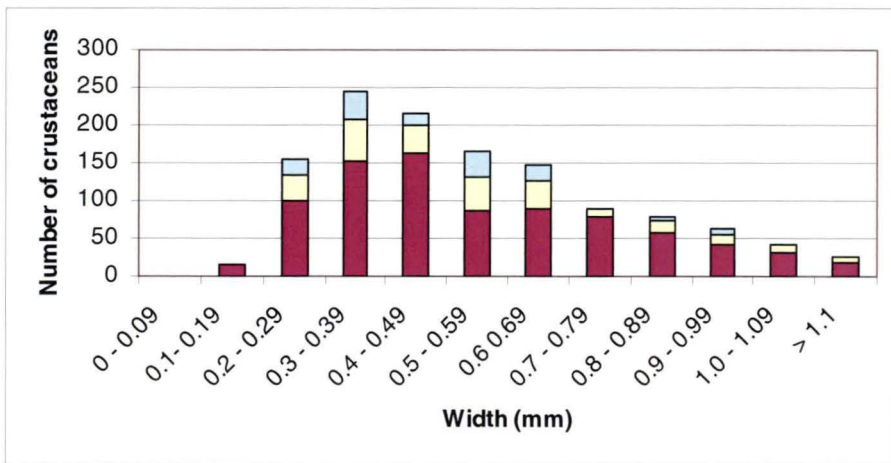


Figure 3.14. Biofouling prey consumed and corresponding net composition. (a) The number of each prey type consumed by 203 day old seahorses and the size of the prey; (b) The number, size and types of prey available on the net panels in April when gut content of 203 day old seahorses was analysed. ■ Harpacticoid copepod, ■ *Hippomedon* sp, ■ *Biribus* sp, ■ *Caprella* sp.

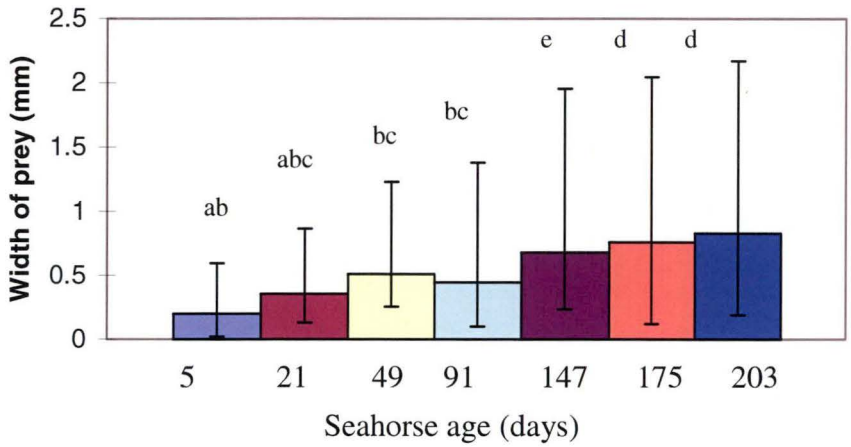
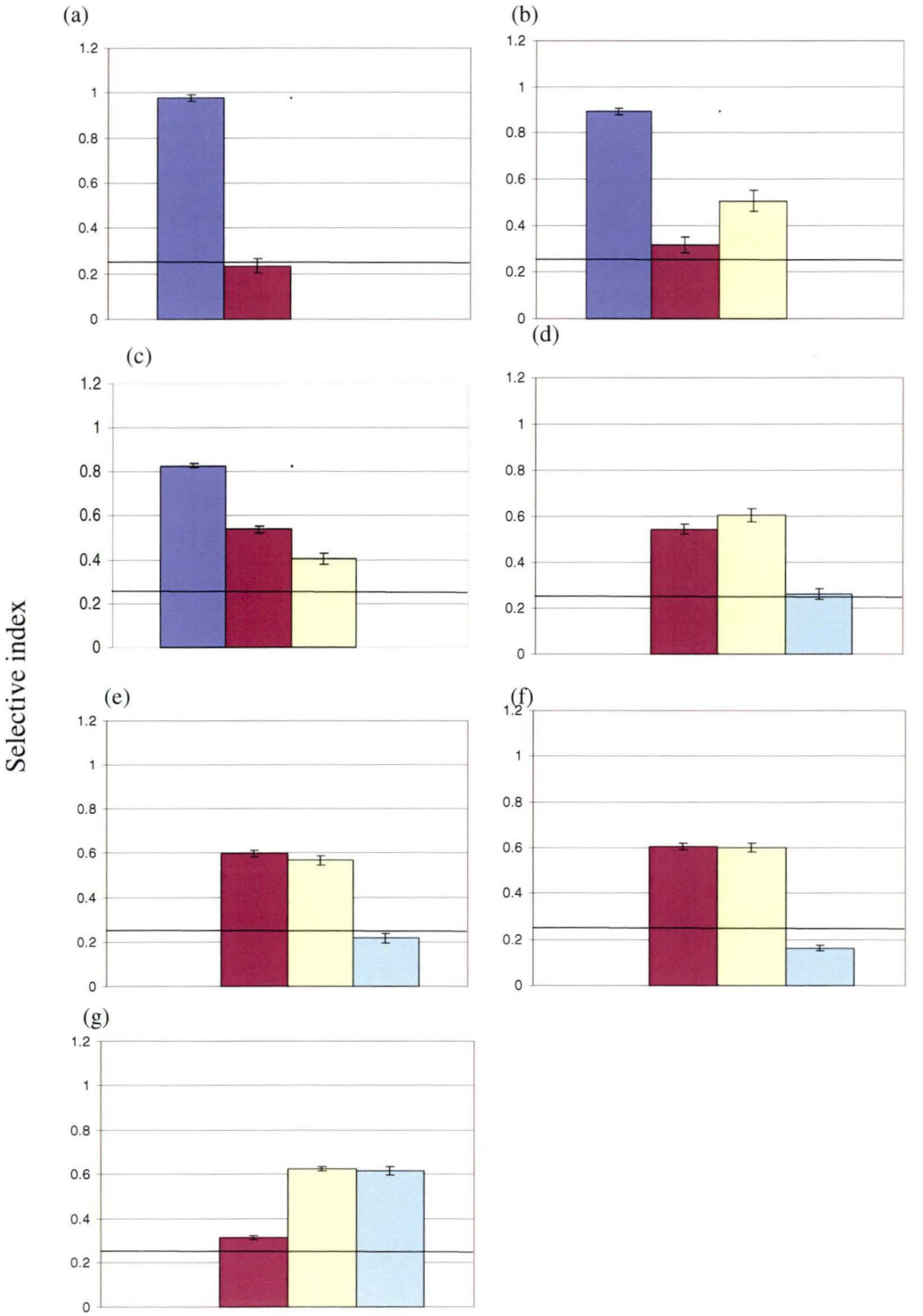


Figure 3.15. The mean ± 5 and 95% quartiles of the prey width consumed by seahorses aged ■ 5, ■ 21, ■ 49, ■ 91, ■ 147, ■ 175 and ■ 203 days old.

Figure 3.16 Mean (± SE) selective index of the main prey items consumed by seahorses (a) selective index for copepods and amphipods consumed by 5 day old seahorses (n = 5 seahorses), (b) selective index for copepods, *Hippomedon* sp and *Biribus* sp consumed by 21 day old seahorses (n = 12 seahorses), (c) selective index for copepods, *Hippomedon* sp and *Biribus* sp consumed by 49 day old seahorses (n = 12 seahorses), (d) selective index for *Hippomedon* sp and *Biribus* sp and caprellids consumed by 91 day old seahorses (n = 12 seahorses), (e) selective index for *Hippomedon* sp and *Biribus* sp and caprellids consumed by 147 day old seahorses (n = 9 seahorses), (f) selective index for *Hippomedon* sp and *Biribus* sp and caprellids consumed by 175 day old seahorses (n = 9 seahorses), (g) selective index for *Hippomedon* sp and *Biribus* sp and caprellids consumed by 203 day old seahorses (n = 9 seahorses). ■ copepod, ■ amphipod 1, ■ amphipod 2, ■ caprellid. (Line represents the α value, 0.25 (based on four potential food items). Values above the α value indicate positive selection).


(a)
(b)
(c)
(d)
(e)
(f)
(g)
Selective index
1.2
1
0.8
0.6
0.4
0.2
0

3.3.3. Effect of copepods on the growth of early juvenile pot-bellied seahorses

Growth of seahorses

There was no significant difference ($F = 0.009$, df 1, $p > 0.05$) between the weight of pot-bellied seahorses in the *Artemia* and copepod treatments at the beginning of the growth trial (3 weeks old) (Figure 3.17, Appendix 9.9). At the completion of the trial there was no significant difference between the weight of seahorses fed *Artemia* enriched with Algamac 3050™ and those fed copepods ($F = 0.054$, df 1, $p > 0.05$) (9 weeks old) (Figure 3.17, Appendix 9.9).

Specific growth rates of seahorses fed *Artemia* enriched with Algamac 3050™ and copepods from the beginning to the end of the feed trial (42 days) were $2.54\%d^{-1}$ and $2.52\%d^{-1}$ respectively.

Condition index of seahorses in the dietary feed trial was determined by examining length to weight relationships. No significant difference ($F = 0.4163$, df 1, $p > 0.05$) was found between the condition of seahorses fed different diets (Table 3.6).

There was little to no variation between the weights and lengths of seahorses in the *Artemia* and copepod treatments at either the beginning or on completion of the trial (Table 3.6).

This trial ran for 6 weeks because the copepod culture started to decline in week five.

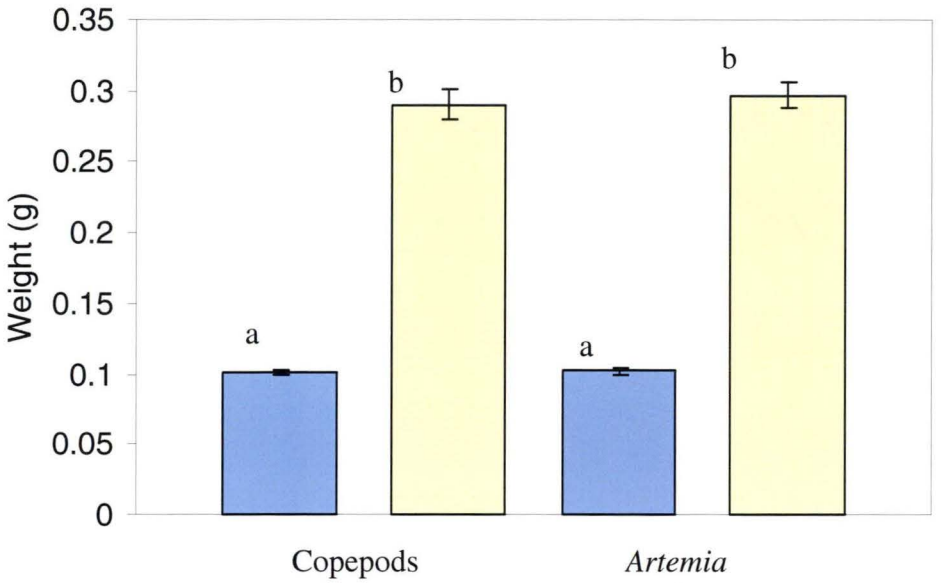


Figure 3.17. Initial (■) and final (■) mean (± S.E) weight of juvenile pot-bellied seahorses fed copepods or *Artemia* enriched with Algamac 3050™. Means which did not significantly differ in the Tukey's HSD test share the same common superscript. (Refer to Appendix 9.9 for further information on the initial and final mean weights).

Table 3.6. Effect of the diet on the length to weight relationship (condition index) of the pot-bellied seahorses and the initial and final coefficient of variation for weight and length for each treatment (a = elevation of the line; b = slope of the line, lower slope = poorer condition; r^2 = explanatory variable).

Condition Index					Coefficient of Variation			
					Weight		Length	
Treatment	a	b	r^2	p	Initial	Final	Initial	Final
Artemia	-0.812	.531	.709	<0.05	0.1540	0.2404	0.0583	0.0898
Copepods	-0.874	.570	.794	<0.05	0.1736	0.2607	0.0657	0.0916

(Regression equation: $\log_{(\chi+1)}$ weight = b $\log_{(\chi+1)}$ length + $\log_{(\chi+1)}$ a)

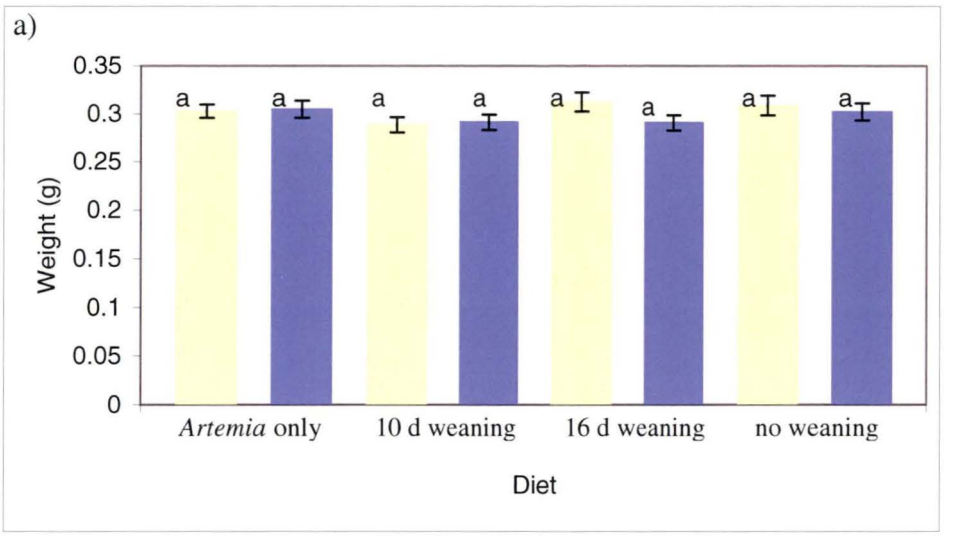
3.3.4. Effect of frozen diets on the growth and condition of seahorses and development of a weaning protocol

Growth of seahorses

There was no significant difference ($F = 0.9437$, df 3, $p > 0.05$) between the mean weight of pot-bellied seahorses in the *Artemia*, frozen amphipod and frozen mysids weaning treatments at the beginning of the growth trial (Figure 3.18, Appendix 9.10). At the completion of the trial there was a significant difference ($F = 83.922$, df 7, $p < 0.05$) between the weight of seahorses fed *Artemia* enriched with Algamac 3050™ and those which were weaned onto frozen amphipods or frozen mysids, with seahorses weaned over 16 days and seahorses fed *Artemia* having significantly higher growth rates then those in the 10 day weaning and no weaning groups (Figure 3.19, 3.20). It was also noted that there was no significant difference in growth between seahorses weaned onto amphipods or mysids.

Over time the rate of seahorse growth changed. In the *Artemia* treatments growth increased relatively steadily. In the one week weaning and no weaning treatments growth was suppressed in the first 3 weeks and then began to increase more rapidly. In the two week weaning treatments growth was suppressed in the first two weeks and then growth rates were similar if not slightly higher than growth rates achieved in the *Artemia* only treatments (Figure 3.19, 3.20).

Specific growth rates of seahorses fed instar II *Artemia* enriched with Algamac 3050™, subjected to an abrupt changeover, weaned on to frozen amphipods over a 10 day period and weaned onto amphipods over a 16 day weaning period were $1.9515\%d^{-1}$, $1.6749\%d^{-1}$,$1.8453\%d^{-1}$, $2.0742\%d^{-1}$, respectively. Specific growth rates of seahorses fed instar II *Artemia* enriched with Algamac 3050™, subjected to an abrupt changeover period, weaned on to frozen mysids over a 10 day period and weaned over a 16 day weaning period $1.9437\%d^{-1}$, $1.7480\%d^{-1}$, $1.8418\%d^{-1}$, $2.1395\%d^{-1}$, respectively.



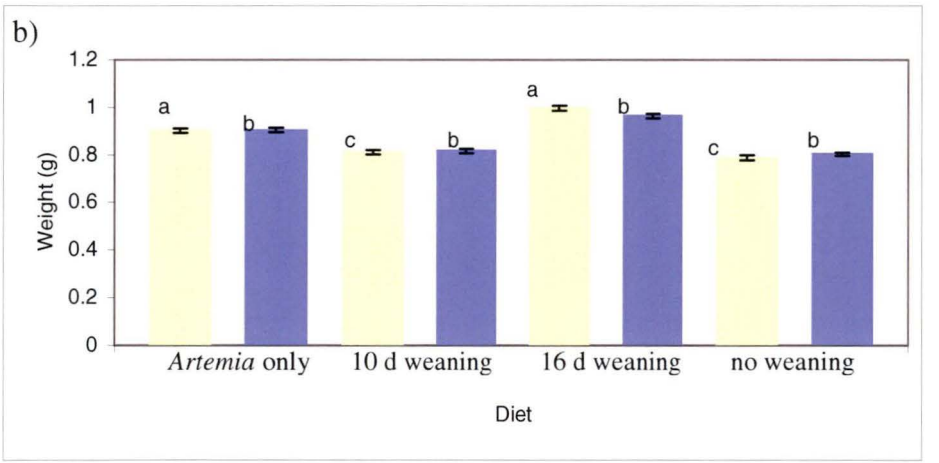


Figure 3.18. (a) Initial and (b) final mean ± S.E weights of fish fed *Artemia*, weaned for 10 days, 16 days and subjected to no weaning period. (■) amphipod fed experiment, (■) mysid fed experiment. (Refer to Appendix 9.10 for further information on initial and final mean weights).

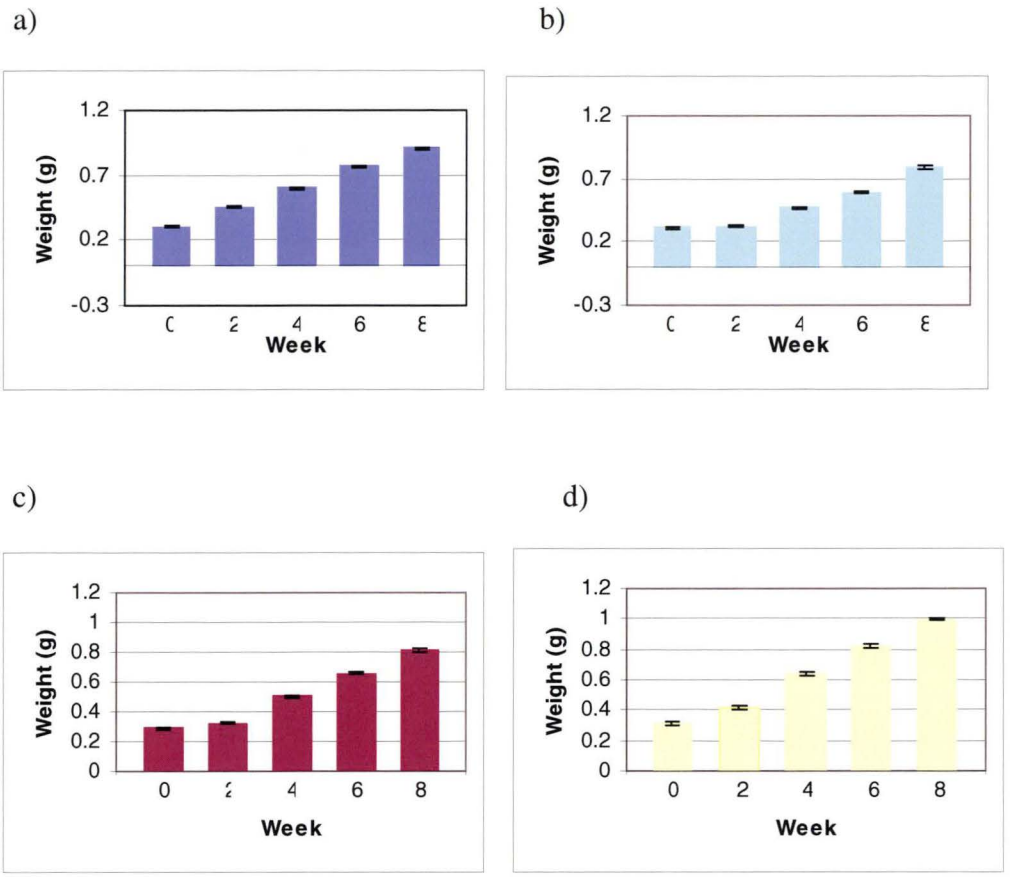


Figure 3.19. Growth of seahorses fed (a) *Artemia*, (b) introduced to frozen amphipods with no weaning period (c) weaned for 10 days on frozen amphipods and (d) weaned for 16 days onto frozen amphipods.

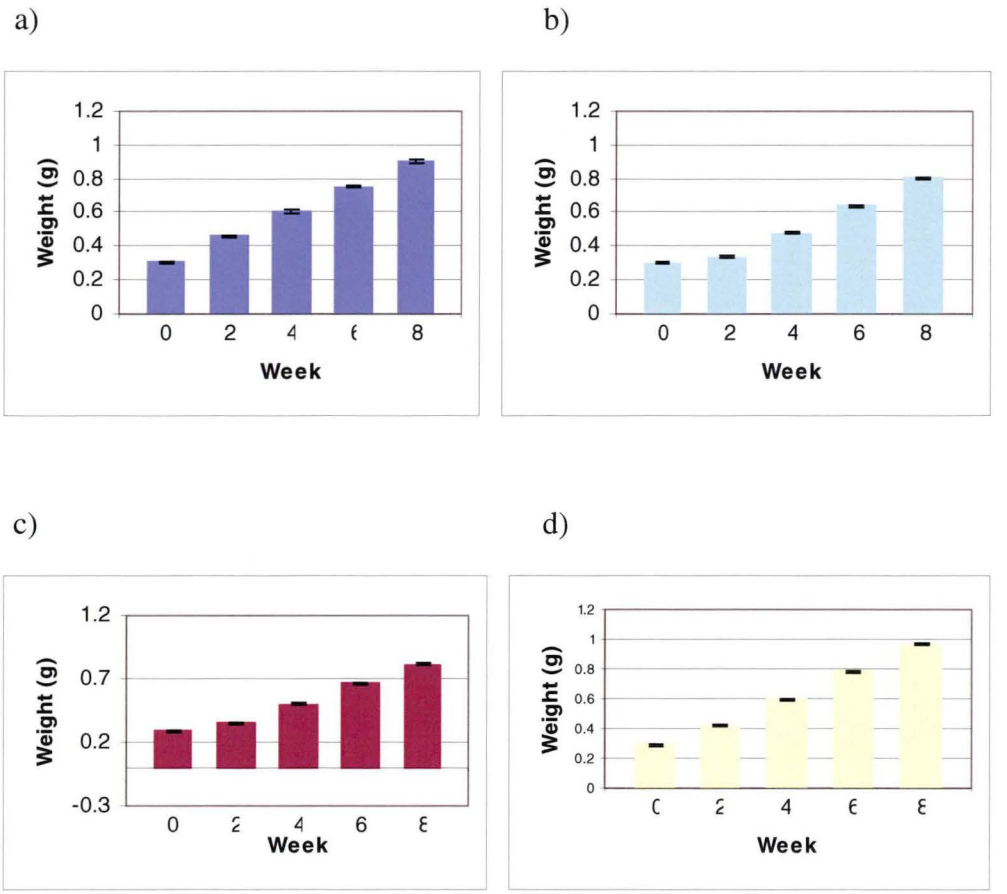


Figure 3.20. Growth of seahorses fed a) *Artemia*, b) introduced to frozen mysids with no weaning period, c) weaned for 10 days on frozen mysids, d) weaned for 16 days onto frozen mysids.

Condition indices of seahorses in the dietary feed trial were determined by analysing final length to weight relationships. No significant difference ($F = 1.784$, df 3, $p > 0.05$) was found between the condition index of seahorses fed *Artemia* or mysids under different weaning periods. In contrast a significant difference ($F = 91.020$, df 3, $p < 0.05$) was found between seahorses fed *Artemia* and those fed amphipods under different weaning periods; seahorses weaned onto amphipods over 16 days had a higher slope (of 1.496) which denotes better condition then seahorses in the other feed treatments (*Artemia*, no weaning, 10 day weaning) (Table 3.7).

There was little to no variation between the weights and lengths of seahorses in the *Artemia* and frozen amphipod and frozen mysid diet treatments at either the beginning or on completion of the trial (Table 3.7).

Feed Intake

Food (dry weight food:wet weight fish) conversion ratios for the frozen amphipod trial were 1.79:1, 1.07:1, 1.27:1, 1.43:1, for *Artemia* only, no weaning period, 10 day weaning period and 16 day weaning period respectively. Food conversion ratio for the frozen mysids trial were 1.9339, 1.1162, 1.8520 and 1.9236, for *Artemia* only, no weaning period, 10 day weaning period and 16 day weaning period treatments respectively. The feed efficiency values for the amphipod trial were 0.78, 0.55, 0.69, and 0.92 for *Artemia* only, no weaning period, 10 day weaning period and 16 day weaning period treatments respectively. The feed efficiency for the mysid trial are 0.89:1, 0.51:1, 0.53:1, 0.51:1 for *Artemia* only, no weaning period, 10 day weaning period and 16 day weaning period treatments respectively.

Table 3.7. Effect of the weaning diets on the length to weight relationship (condition index) of the pot-bellied seahorse and the initial and final coefficient of variation for seahorse weight and length in each weaning treatment (a = elevation of the line; b = slope of the line, lower slope = poorer condition; r^2 = explanatory variable).

Condition Index					Coefficient of Variation			
					Weight		Length	
Treatment	a	b	r^2	p	Initial	Final	Initial	Final
Mysid trial								
Artemia only	-1.095	0.716	0.622	<0.05	0.0878	0.2529	0.0663	0.0923
10 day weaning	-1.035	0.689	0.451	<0.05	0.0855	0.2209	0.0770	0.0823
16 day weaning	-1.577	0.889	0.611	<0.05	0.0812	0.2393	0.0684	0.0931
No weaning	-0.642	0.552	0.376	<0.05	0.0630	0.2337	0.0713	0.0817
Amphipod trial								
Artemia only	-0.595	0.544	0.525	<0.05	0.0842	0.2596	0.0698	0.1042
10 day weaning	-1.385	0.813	0.603	<0.05	0.0862	0.2195	0.0652	0.0848
16 day weaning	-3.320	1.496	0.634	<0.05	0.0874	0.2615	0.0706	0.0993
No weaning	2.406	0.541	0.717	<0.05	0.1037	0.1943	0.0713	0.0818

(Regression equation: $\log_{(\chi+1)}$ weight = b $\log_{(\chi+1)}$ length + $\log_{(\chi+1)}$ a)

In both the frozen amphipod and frozen mysid weaning trials it was found that seahorses fed enriched *Artemia* consumed between 92 to 98 % of the *Artemia* present in the morning and 91 to 98 % of *Artemia* present in the afternoon throughout the trial (Figure 3.21 and 3.22). When amphipods were abruptly introduced it was found that seahorses consumed 10 to 20 % of the amphipods offered in the morning and 20 to 30 % in the afternoon during the first three days of the trial. The amount consumed increased slowly with more being consumed in the afternoon until consumption started to plateau at around day 8. Around day 15 there was a sudden increase in consumption, which correlates with an increase in the amount of feed offered after readjusting feed quantities following weight checks. From day 15 onwards consumption of amphipods in the morning and afternoon was fairly similar (Figure 3.23). When mysids were introduced abruptly (ie no changeover period) it was found that seahorses consumed around 50% of the mysids present in both the morning and afternoon feeds from day 1 of the weaning period (Figure 3.24).

When seahorses were weaned onto both frozen mysids and amphipods over a 10 day period it was found that the frozen diets were not consumed on the first day; around 20% of the frozen diet present was consumed on the second and third day and when *Artemia* was removed from the morning meal on day 3 the amount of frozen diet consumed increased slowly until day 7 when consumption started to plateau. During this time seahorses were still being fed *Artemia* in the afternoon, of which they consumed up to 98% daily. When the frozen diet was introduced into the afternoon meal on day 7 around 45% of the frozen diet was consumed and on day 10 when *Artemia* was completely removed from the diet, frozen diet consumption increased and continued to increase slowly until around day 15. On day 15, consumption of the frozen diets in both the morning and afternoon feeds started to plateau and consumption remained slightly higher in the afternoon meal than in the morning meal (Figure 3.25, 3.26).

When seahorses were weaned onto frozen diets over a 16 day period it was found that frozen diets were not consumed on the first day. During the next four days they consumed around 25% of the frozen diet and around 95% of the *Artemia* present (Figure 3.27, 3.28). On day 6 when *Artemia* was removed from the morning meal frozen diet consumption increased to around 30% and continued to increase slowly until around day 16 when consumption was approximately 65%. During these 15 days seahorses were fed *Artemia* in the afternoon and they consumed around 97% of the *Artemia* present each day. On day 11 when frozen diets were introduced into the afternoon meal seahorses consumed around 40% of the frozen diets and this consumption rate remained fairly similar. It was also noted that consumption of the frozen diets tended to be higher in the afternoon meal than the morning meal.

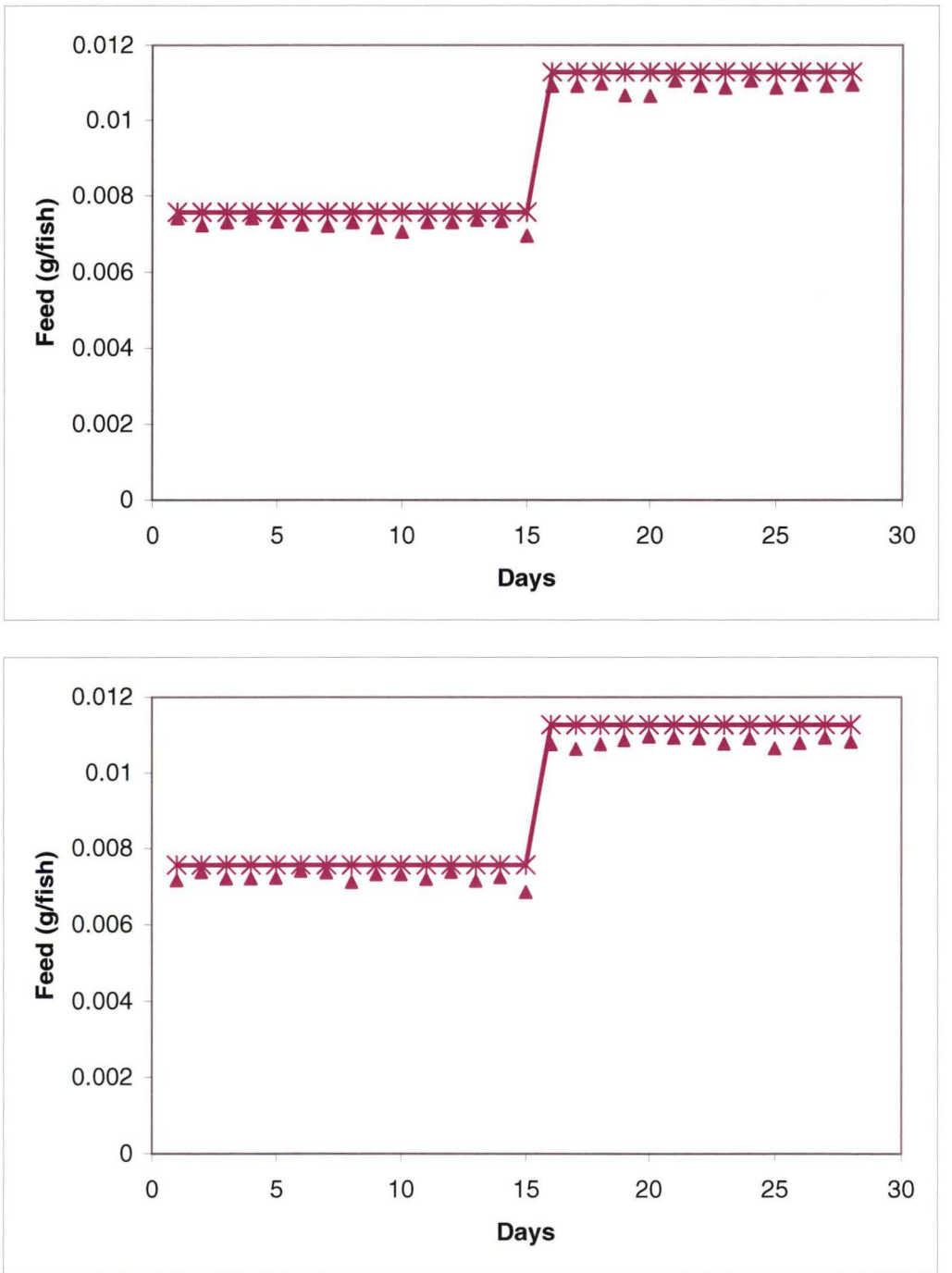


Figure 3.21. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the *Artemia* control treatment in the amphipod trial. x amount of *Artemia* offered, ▲ amount of *Artemia* consumed.

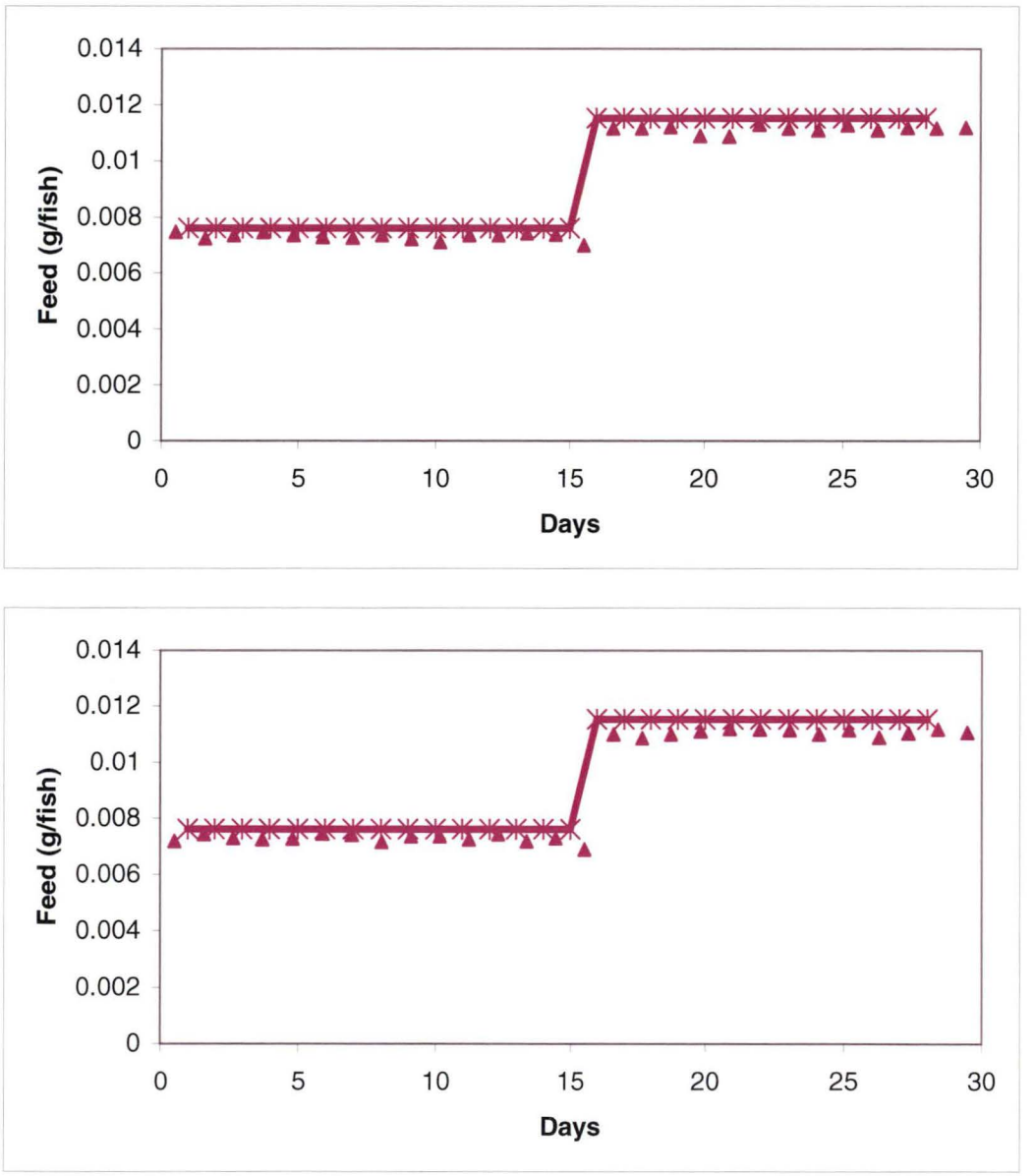


Figure 3.22. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the *Artemia* control treatment in the mysid trial. x amount of *Artemia* offered, ▲ amount of Artemia consumed.

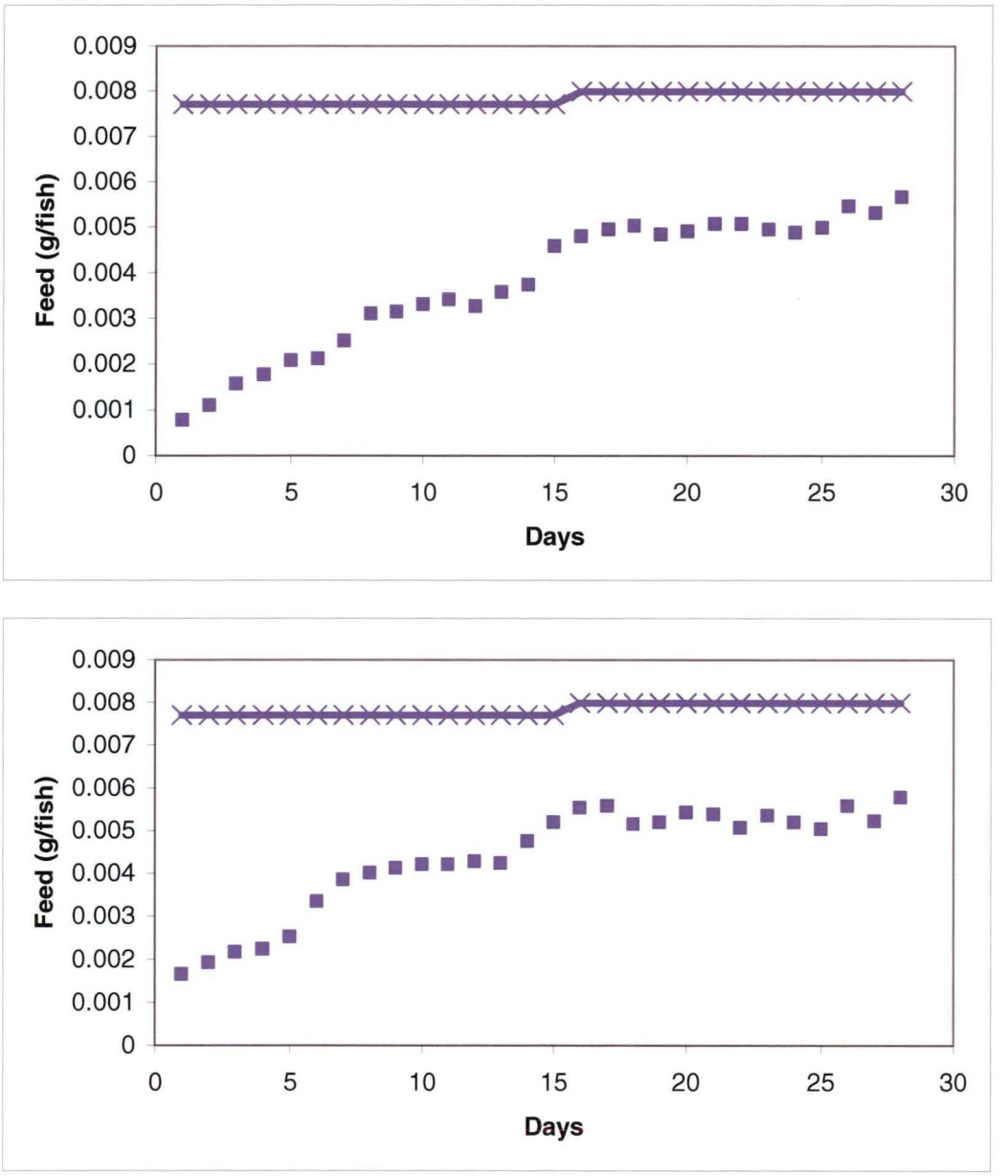


Figure 3.23. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the no weaning period treatment in the amphipod trial. x amount of amphipods offered, ■ amount of amphipods consumed.

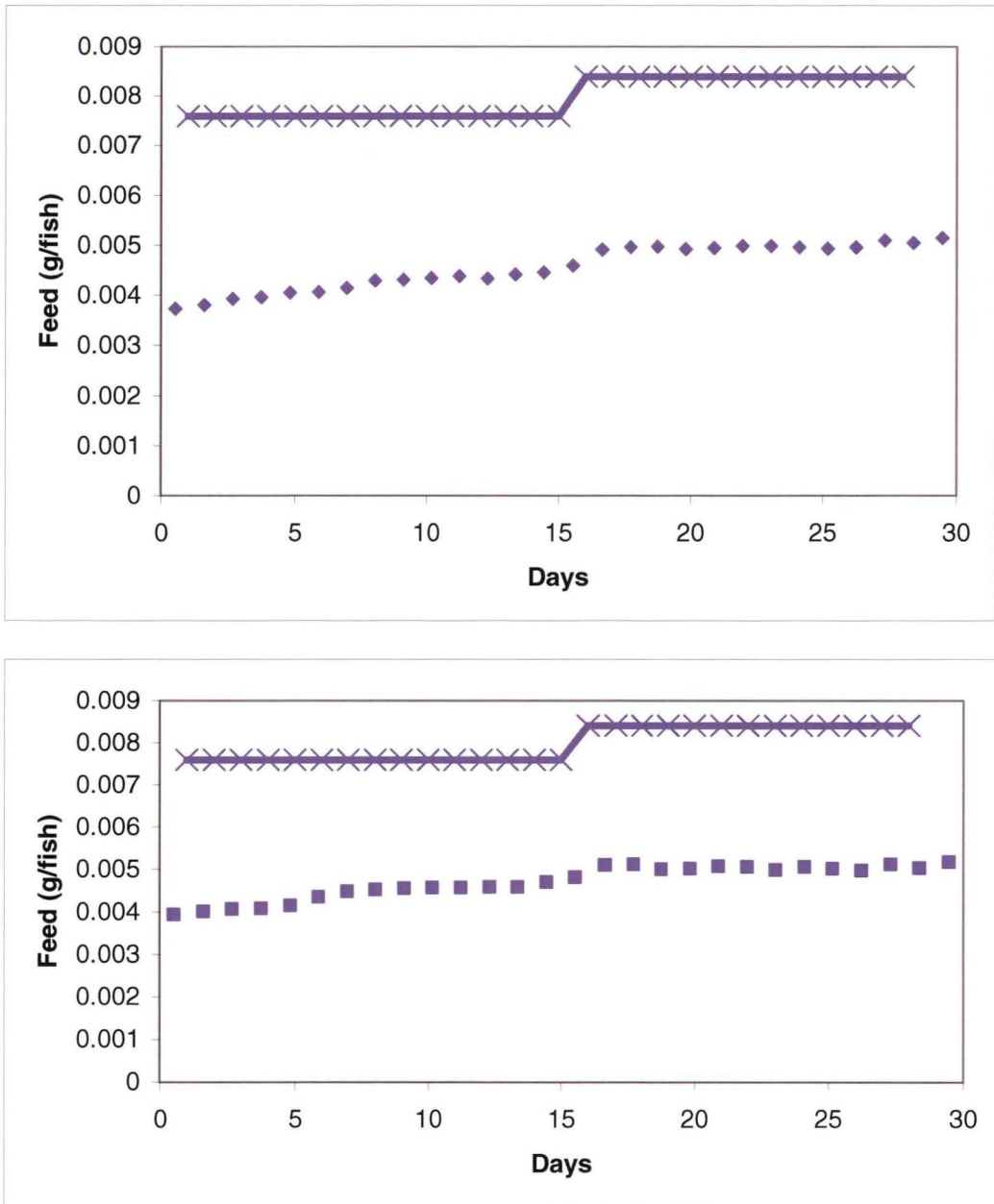


Figure 3.24. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the no weaning period treatment in the mysid trial. x amount of mysids offered, ■ amount of mysids consumed.

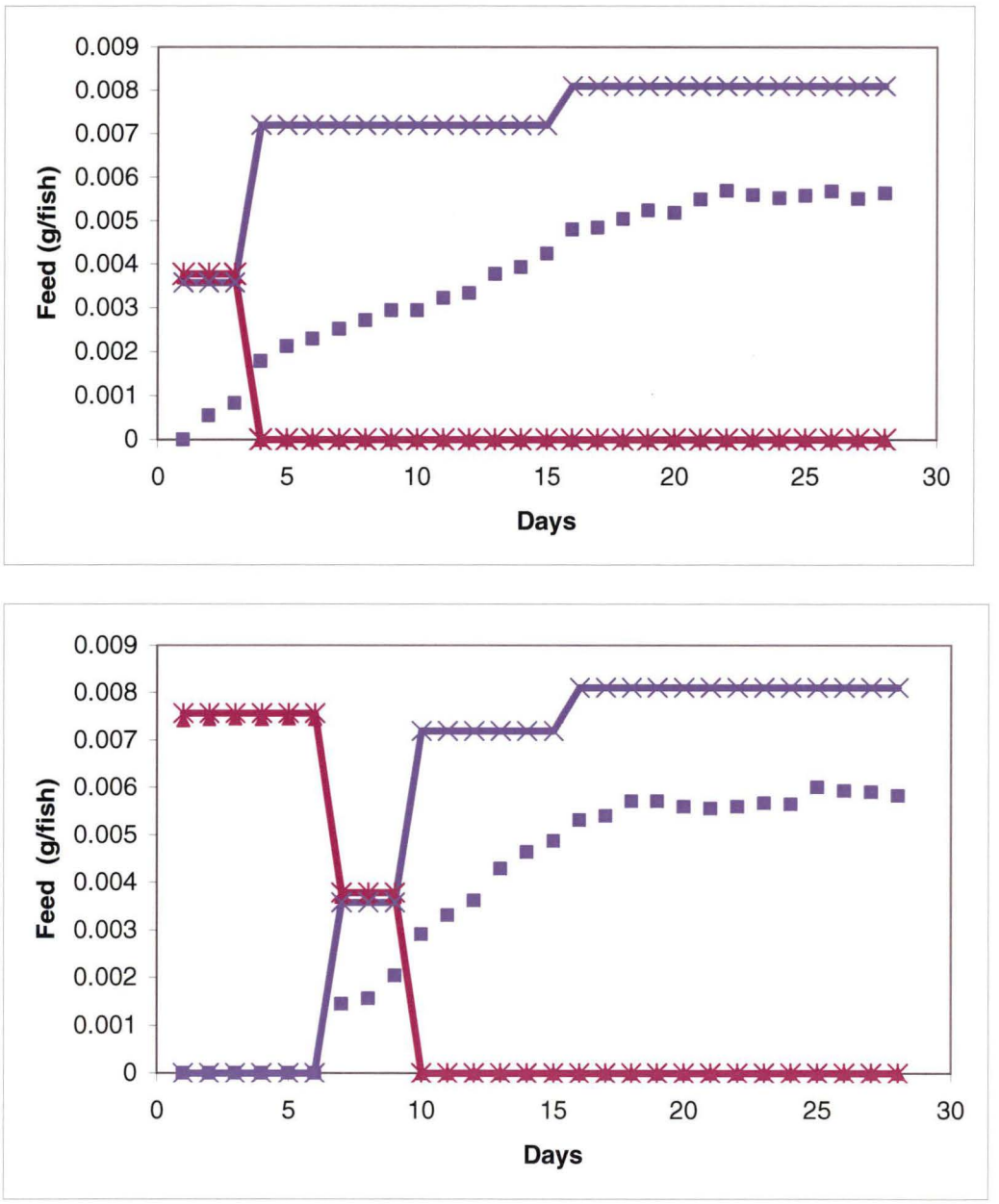


Figure 3.25. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the 10 day weaning period treatment in the amphipod trial. x amount of amphipods offered, ■ amount of amphipods consumed, x amount of *Artemia* offered, ▲amount of *Artemia* consumed.

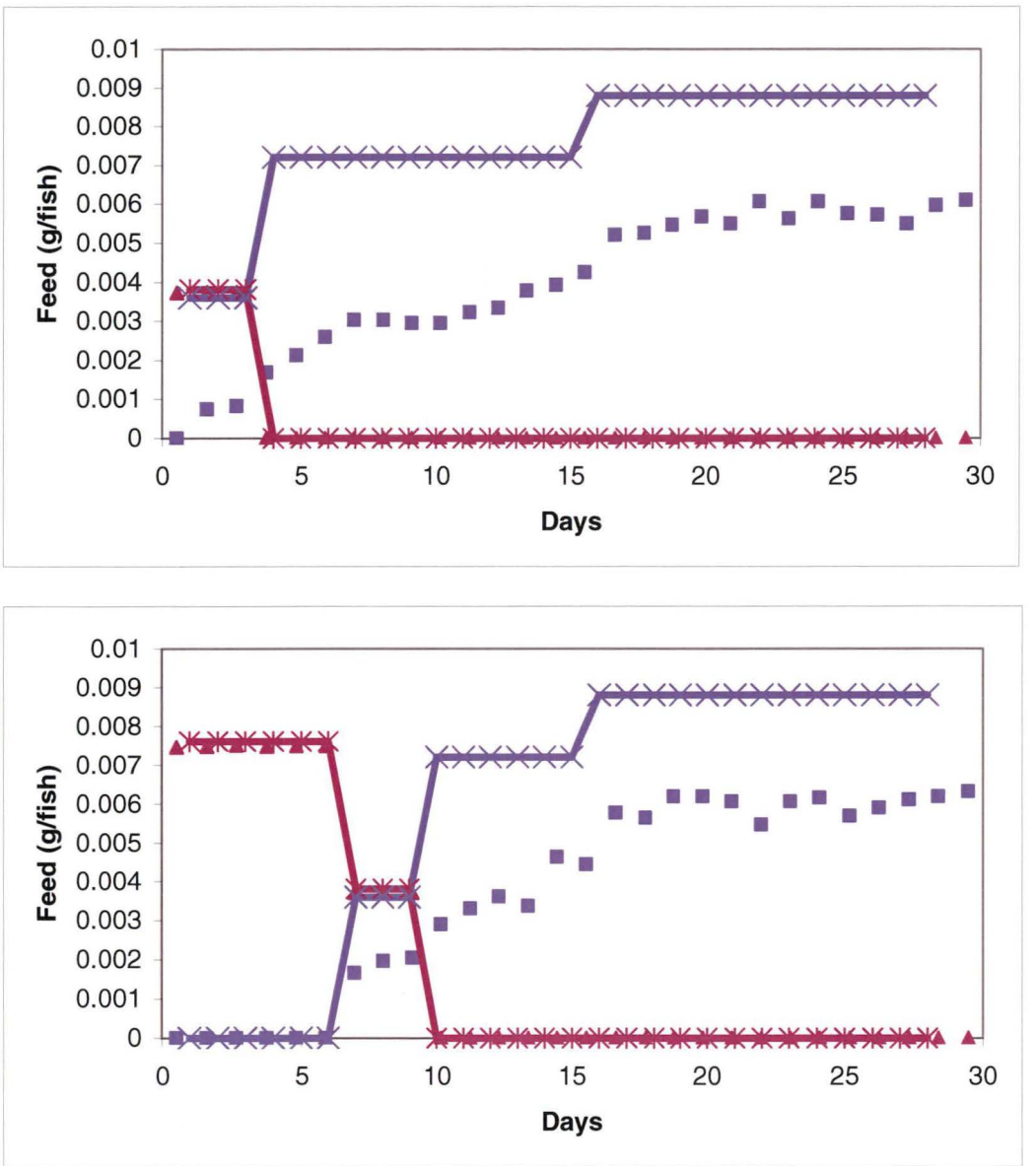


Figure 3.26. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the 10 day weaning period treatment in the mysid trial. x amount of mysids offered, ■ amount of mysids consumed, x amount of *Artemia* offered, ▲amount of *Artemia* consumed.

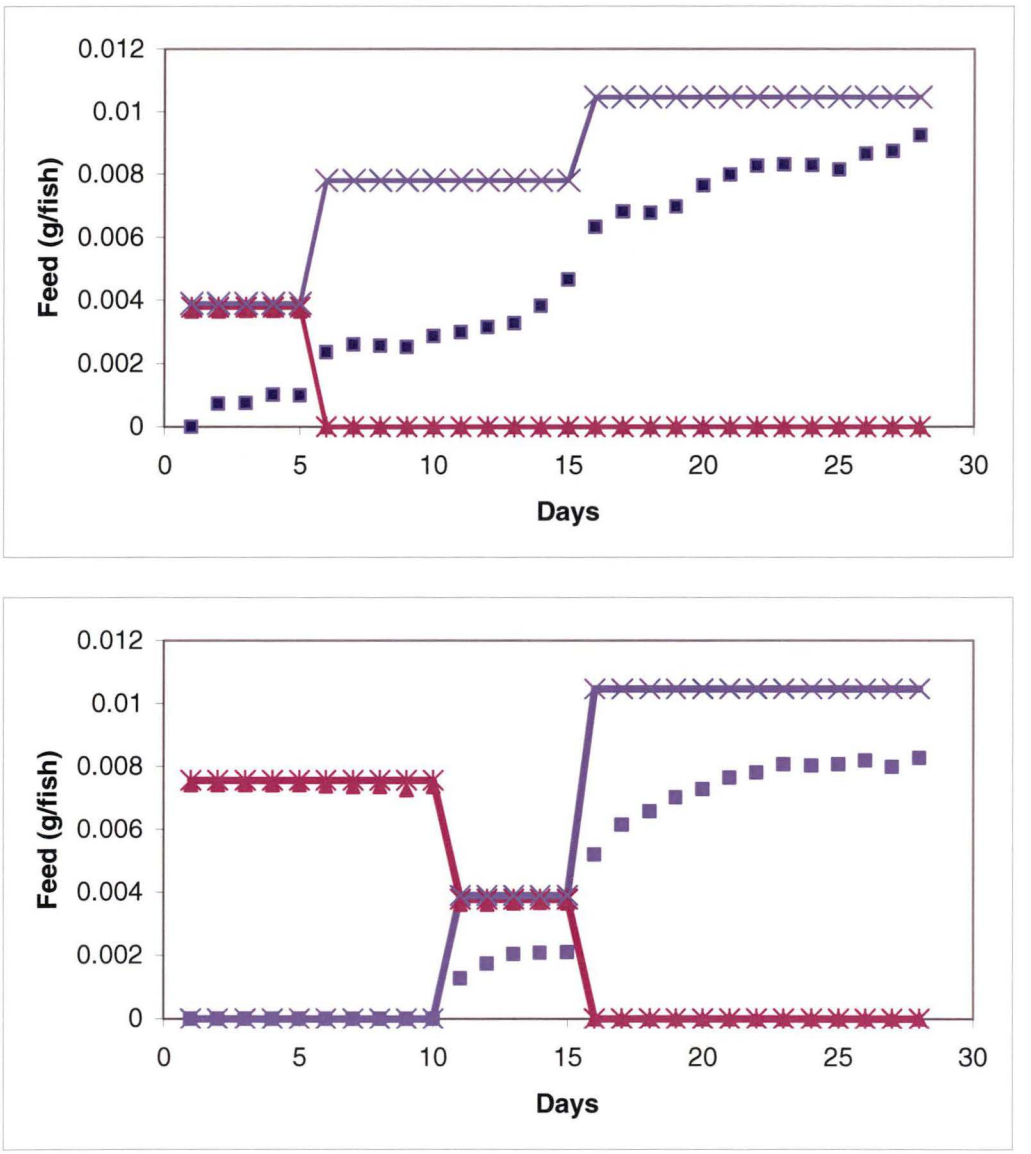
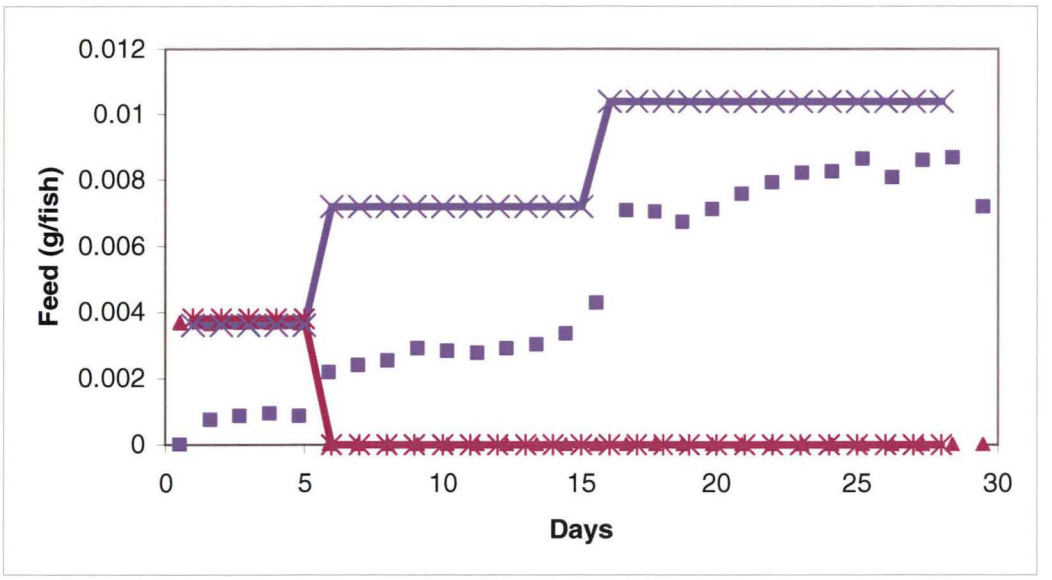


Figure 3.27. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the 16 day weaning period treatment in the amphipod trial. x amount of amphipods offered, ■ amount of amphipods consumed, x amount of *Artemia* offered, ▲amount of *Artemia* consumed.



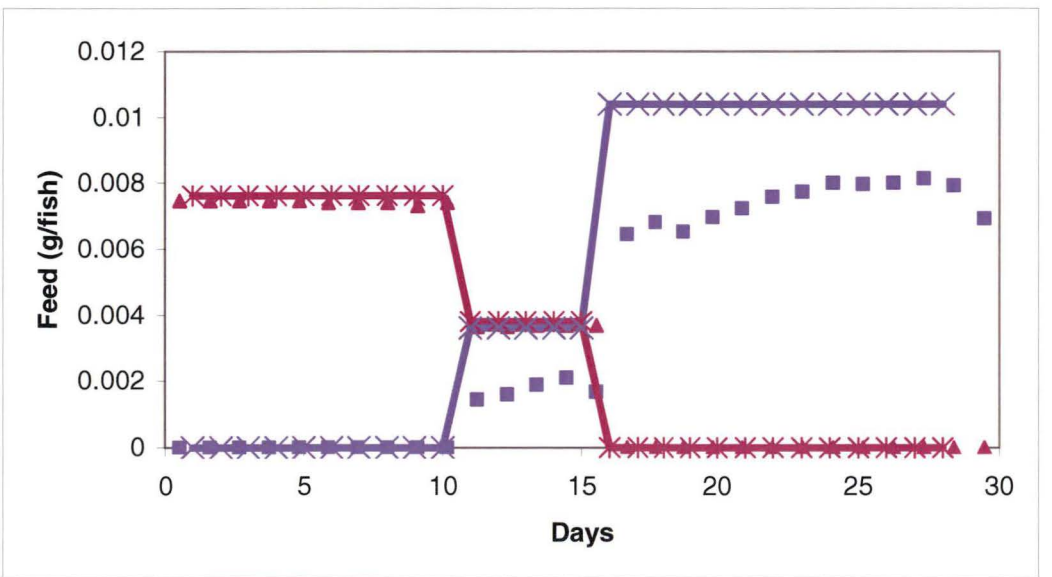


Figure 3.28. Morning (a) and afternoon (b) feed intake (g/fish) for seahorses in the 16 day weaning period treatment in the mysid trial. x amount of mysids offered, ■ amount of mysids consumed, x amount of *Artemia* offered, ▲ amount of *Artemia* consumed.

3.4 DISCUSSION

This study showed that the growth and survival of seventeen week old seahorses fed biofouling crustaceans and *Artemia* enriched with Algamac 3050™ was similar and newborn, 3 week old, 7 week old and 13 week old seahorses readily consumed crustaceans from the biofouling. It was also found that the growth of three week old seahorses fed copepods had similar growth and survival to those fed enriched *Artemia*, and thirteen week old seahorses weaned onto frozen amphipods and frozen mysids over a 16 day weaning period had similar growth to seahorses fed enriched *Artemia*. These results suggest that the reliance of *Artemia* in the culture of pot-bellied seahorses could be reduced by totally or at least partially replacing *Artemia* with alternative live feeds such as biofouling crustaceans or copepods in younger seahorses and weaning seahorses from thirteen week onwards onto frozen diets.

Alternative live feeds and prey selection

Of the organisms present in the biofouling pot-bellied seahorses consumed harpacticoid copepods, 2 species of gammarid amphipods (*Hippomedon* sp and *Biribus* sp) and caprellids (*Caprella* sp). Copepods were consumed by newborns and 3 and 7 week old seahorses, amphipods were consumed by 3, 7, 13, 21 and 25 week old seahorses and the larger 25 and 29 week old seahorses consumed amphipods and caprellids. Similar findings were found in the lined seahorse (*Hippocampus subelongatus*) as their diet consisted of gammarid and caprellid amphipods and carid shrimp (Wong & Benzie, 2003). As these crustaceans generally 'crawl' or 'walk' on the sediment or phytal surface (Hicks & Coull, 1983; Main, 1987; Ryer, 1988) they are available to the seahorses at the ages tested as they show sedentary feeding behaviour. Supporting this are other studies on the Sydney seahorse (*Hippocampus whitei*) and the dwarf seahorse (*Hippocampus zosterae*), which were found to feed mostly on amphipods and harpacticoid copepods respectively (Burchmore et al., 1984; Tipton & Bell, 1988).

This study shows that prey size selected by the pot-bellied seahorse increases with age. Newborns (TL 15. 7 ± 0.5 mm) consumed mainly copepods (width 0.143 - 0.321 mm) and a few amphipods (*Hippomedon* sp) (width 0.211 - 0.404); three week old seahorses (TL 25.3 ± 0.5 mm) consumed both copepods (widths 0.168 - 0.274 mm) and *Hippomedon* sp (width: 0.253 - 0.386 mm); seven week old seahorses (TL 36.6 ± 0.6 mm) consumed mainly *Hippomedon* sp (width 0.197 - 0.727 mm) and a small amount of copepods; thirteen week old seahorses (TL 50.2 ± 0.9 mm) consumed mostly *Hippomedon* sp (width: 0.254 - 1.0615 mm), a moderate number of *Biribus* sp (width: 0.254 - 1.06 mm) and a few *Caprella* sp (width: 0.6373 - 1.265 mm); twenty five week old seahorses (TL 79.5 ± 2.6 mm) consumed mainly *Hippomedon* sp (width: 0.356 - 1.481 mm), and a number of *Biribus* sp (width: 0.33 - 1.349 mm) and *Caprella* sp (width: 0.604 - 0.83 mm); and twenty nine week old seahorses (TL 84.6 ± 2.7 mm) consumed mainly *Hippomedon* sp (width: 0.446 - 1.695 mm), and a number of *Biribus* sp (width: 0.33 - 1.349 mm) and *Caprella* sp (width: 0.497 - 1.294 mm).

This increase in prey size selection with age is common for many fish species. For example, there was a significant relationship between the size of blacktail comber (*Serranus atricauda*) and its prey (Morato et al., 2000), the composition and relative importance of prey species changed to include progressively larger prey in the California halibut (*Paralichthys californicus*) (Wertz & Domeier, 1997) and the prey size of pike increased with pike length, however it was also noted that the minimum size of prey did not increase as fast as the maximum size and this is because pike are opportunistic feeders (Kahilainen & Lehtonen, 2003). Consumption of prey by predators is limited by gape size (Kahilainen & Lehtonen, 2003; Mittelbach & Persson, 1998) and the ability to ingest, handle and capture larger prey comes about with age. With age, gape size increases, sensory function increases and locomotor ability improves. In the case of seahorses, newborns tend to be buoyant near the surface; after a few days they start holding on to the

substrate and it is not until they are about three to four weeks old that they start to position themselves on the bottom of the tanks (personal observation). This study shows that as seahorses grow the consumption of small prey items like the copepods decrease, while consumption of larger species like caprellids and *Biribus* sp increase. As prey size was shown to increase with seahorse body length it can be suggested that large seahorses eat large prey. This would of course be of benefit as it would be assumed that larger seahorses have a higher energy demand and it would maximise the energetic gain relative to capture effort (Elliot & Hurley; 2000; Hughes, 1997; Morato et al., 2000) however it is evident that seahorses did not necessarily select prey on the basis of the largest possible size they are capable of handling. While the upper range of prey size increased with predator size, the minimum prey size remained relatively constant. Seahorses, particularly the older seahorses, consumed prey from a number of class sizes and this probably allows them to utilise all the available resources. Other studies on prey selection have found that fish which exhibit a high degree of size-specific prey selection often select prey species that are considerably smaller than their maximum mouth gape would allow (Hansen & Wahl, 1981; Mills et al., 1986; Platell & Potter, 1999; Shaw et al., 2003). Older seahorses were found to consume prey from a number of size classes, thus it can be said that seahorses do not select their prey based solely on size suggesting they are opportunistic feeders. The narrower range of prey sizes selected by the younger seahorses may be limited by their gape size and types of crustaceans of that size.

In this study both amphipod species were similar in size but *Hippomedon* sp. was the main prey consumed by every age group studied except newborns. Studies have shown that selection of an individual prey type among a number of others of similar size provides evidence for selection based on other prey characteristics. Possible species-specific prey characteristics that may influence prey selection by the seahorses include prey motion and prey form (eg number and size of legs and body colour), as studies with other fish have shown that motion is a key factor in

determining selection (Campbell, 1991; Crowl, 1989; Zaret, 1980). It is therefore possible that the different movement and activity patterns displayed by the live prey species in this study may have had an affect on prey selection. Other possible mechanisms affecting prey selection are prey colour (Utne, 1997; Utne-Palm, 1999), relative abundance of the prey species as predation can quite often be directed at the most abundant and available prey species (Bohn et al., 2002; Hughes, 1997; Mann, 1982; Naesje et al., 1998; Niva & Julkunen, 1998), foraging efficiency of the predator and the prey (Turesson et al., 2002) and defence adaptations/escape mechanisms of the prey (Houde & Schekter, 1980; Reiriz et al., 1998; Ritz, 2000; Ritz et al., 1997; Turesson et al., 2002). In this study it was found that *Hippomedon* sp. was the most abundant crustacean present on the net panels in every month studied. However, much further work is required to determine why *Hippomedon* sp. was the preferred choice of seahorses.

Frozen diets and weaning protocol

The weaning trial demonstrated that seahorses successfully grew on both the frozen amphipod and frozen mysid diets. The ability of seahorses to grow successfully on frozen diets was also shown by Woods & Valentine (2003) who found that after 3 months there was no difference in seahorse length, weight or condition factor when 10 month old seahorses were fed a daily ration of live *Artemia* enriched with Algamac 3050™, frozen mysids (*Amylops kempi*) or a combination of live *Artemia* and frozen mysids (50/50). In the present study it was found that the growth of 21 week old seahorses weaned onto frozen diets over 16 days grew significantly better than seahorses weaned over a 10 period and those subjected to an abrupt changeover. It was also noted that on completion of the trial seahorses weaned over a 16 day period exhibited similar growth to seahorses fed enriched *Artemia*. Thus, while this experiment demonstrates that frozen diets can be used as an alternative food for seahorses it also demonstrates that seahorses require a period of co-feeding to recognize and accept a new diet, because it was found that feed intake and, as a consequence, growth lagged at first and then increased over the trial. It was also

seen that a longer period of co-feeding (16 days) gave rise to better growth in seahorses. This need for a longer co-feeding period by seahorses was also seen by Shapawi (2001) who noted that seahorses grew better when introduced to food over a two week period.

Giant seabass (*Stereolepsis gigas*) and gilthead sea bream (*Sparus aurata*) showed improved growth on dry feeds when dry feed was offered as a supplement for several days prior to the start of weaning (Rosenlund et al., 1997). Larvae fed a mixed diet of *Artemia* and dry food produce better specific growth rates and survival relative to larvae fed exclusively on *Artemia* or dry feed (Garcia-Ortega et al., 2003; Petkam & Moodie, 2001; Verreth & Tongeren, 1989). For example, Holt (1993) achieved 60% survival in red drum fed a commercial diet supplied in combination with live food for the first 5 days and Hart & Purser (1996) showed that greenback flounder (*Rhombosolea taprina*) could be successfully weaned before metamorphosis using a 10 day co-feeding period. Similarly Canavate & Fernandez-Diaz (1999) found growth was enhanced in Senegal sole (*Solea senagalensis*) larvae when co-fed the artificial diet with live food and Southern flounder (*Paralichthys lethostigma*) weaned gradually exhibited better growth rates than those subjected to no weaning period (Bengston et al., 1999; Daniels & Hodson, 1999). All these studies have indicated that learning to accept a new diet (criteria including feeding capture success, latency or feeding duration and handling effort) takes times and acceptance variously occurred after 1 - 20 days of exposure to the new prey. In contrast some studies have shown that fish do not eat new diets at all in the presence of original diets or until *Artemia* are seriously reduced (Bengston et al., 1999; Baskerville-Bridges & Kling, 2000a; Daniels & Hodson, 1999; Koven et al., 2001). In general a period of co-feeding is the preferred option as it increases the acceptability of a new diet when the previous feed is withdrawn by allowing the fish a pre-conditioning phase. It has also been found that fish which have had prior experience of a particular prey type will show improved feeding upon the same prey when it is next encountered, that is, fish positively

select familiar prey types and once they start on a diet, they will continue to consume that diet (Cox & Pankhurst, 2000; Dutton, 1992; Guthrie et al., 2000; Rosenlund et al., 1997).

The success of weaning can be affected by a number of issues including the age of fish, their feeding behaviour, duration of the changeover period, the physical properties of the diet (colour, texture, buoyancy) and its size (Daniels & Hodson, 1999; Khemis et al., 2003; Petkam & Moodie, 2001; Rosenlund et al., 1997). This study suggests that a weaning period of around 16 days is required, however it could be that the growth of seahorses weaned for 10 days and even the seahorses fed frozen diets without co-feeding may have eventually caught up in growth. It can be argued that the initial slow growth is of little concern as fish will catch up later and that by weaning for shorter periods or by changing the diet over abruptly is advantageous as it reduces the production costs by shortening the period that *Artemia* is required. Daniels and Hodson (1999) reported that although younger fish grow better when fed *Artemia*, fish fed dry feeds for 2 - 3 months grow faster than those fed *Artemia*; thus the lower growth of young fish fed a dry diet can be recovered after a few months. Hart and Purser (1996) found that a 20 day weaning period appeared to result in the best growth and highest survival, but a 10 day overlap may result in reduced *Artemia* cost with minimal effect of performance. Extending the weaning period beyond 10 days increases the costs associated with the provision of *Artemia* and delays the onset of mortality without improving the survival rate. The increased growth rate might offset the increase in production costs but further research is required to quantify this. In order to wean 91 day old seahorses onto frozen diets a longer co-feeding period is recommended as better growth rates are achieved, however, further studies may show that fish subjected to a shorter weaning period may eventually show growth compensation.

This study successfully weaned 91 day old seahorses onto both frozen amphipods and mysid diets, however to further reduce the dependence on live feeds which are only seasonally available and are a potential source of pathogens (Petkam &

Moodie, 2001) fish should be weaned onto inert diets as early as possible (Hamlin & Kling, 2001). Woods & Valentine (2003) suggested that the mysids used in their study were 10 mm in length, and while acceptable by fish from 80 mm standard length onwards, they were too large for younger seahorses. In this study 91 day old seahorses were chosen based on the finding that in the biofouling trial, seahorses that were 91 days old (50 mm total length) and older fed on caprellids (5.5 - 7.2 mm long, 0.74 - 0.85 mm wide) while younger seahorses did not eat these longer prey types. The earliest weaning time is somewhat restricted by the size range of prey. Unless small mysids are specifically caught mysids are probably not an ideal choice for an early weaning diet as although purchased mysids may range in size they would by easily damaged during grading. Amphipods on the other hand are relatively easy to grade and based on this study the smallest size of amphipods collected were on average 0.321 mm in width and all ages of seahorses examined in this study were shown to consume this prey size.

If early juvenile seahorses can be weaned on to an inert diet it may still be essential to supplement the diet with a live feed component as live feeds may actually stimulate feeding and aid in digestion (Teshima et al., 2000; Woods & Valentine, 2003; Yufera et al., 2000). Also, as seahorses are initially pelagic feeders, young juveniles may require live feeds as they remain in the water column. In the case of feeding biofouling crustaceans it may be necessary to continue to feed seahorses on *Artemia* in the first few weeks of culture as components of the wild zooplankton may be too large and actually capable of harming the seahorses.

In conclusion, the growth and condition of seahorses fed alternative live and frozen diets was similar to those fed enriched *Artemia*, there was a strong predator prey relationship with prey size increasing with predator size, seahorses attained their final body shape at around 91 days of age and seahorses require a long co-feeding period when weaning onto a novel diet. This study has described a range of diet sizes suitable for different aged seahorses, which will be useful in weaning trials

and as a guide for finding alternative diets. As older seahorses were able to utilise all resources in that they consumed prey over a broad size range, it can be said that seahorses, particularly the older ones, are opportunistic feeders. The findings also suggest that, as seahorses may choose prey based on factor(s) other than size, there is scope for improving prey consumption through manipulation of other prey characteristics such as colour, motion and shape and younger seahorses could be weaned onto frozen diets if the appropriate size feed is available.

It would have been ideal to conduct the two month growth trial, which was set up to determine whether seahorses fed biofouling crustaceans had comparable growth to those fed on *Artemia*, on younger fish. However, it was unknown at the time whether seahorses could exist solely on a biofouling diet. Based on the fact that the seahorses appeared to be doing well during the trial it was decided to determine whether younger seahorses would feed off the biofouling panels. Due to restrictions on tank space, numbers of net panels and logistics of feed collection 20 fish of five different age classes (newborn, 3, 7, 9 and 13 week olds) were fed biofouling crustaceans for two weeks. Although further growth trials are required to determine whether younger seahorses can persist long term on a biofouling diet it was found that all age classes readily consumed organisms out of the biofouling. Thus from the results of this study possible feeding regimes for seahorses in culture with a reduced dependence on *Artemia* include:

1) Feed newborns *Artemia* for the first 2 weeks, copepods and then wean at 13 weeks onto a frozen diet.
2) Feed newborns *Artemia* for the first 2 weeks, biofouling and then wean at 13 weeks onto a frozen diet.
3) Feed newborns copepods for the first month, biofouling and then wean at 13 weeks onto a frozen diet.
4) Feed newborns biofouling from newborn onwards and then wean at 13 weeks onto a frozen diet.

5) Feed newborns a live diet and then wean at an earlier age with an appropriate sized frozen diet.

The use of alternative live feeds to replace *Artemia* in the culture of pot-bellied seahorses is not the preferred option as all live feeds are plagued with similar problems (potential source of contamination and subject to seasonal variation) and government regulations may restrict the species which can be cultured and permits are required to collect wild stock. Biofouling crustaceans are difficult to remove from the net (collection panel) without causing damage to them. As a result, the live crustaceans cannot be sorted and there can be organisms present that could potentially harm pot-bellied seahorses. While culture of alternative live diets would ensure the availability of the diet, production remains uncertain, for example, the culture of copepods tends to be unstable and prone to "crashing" as seen in this study. Thus although alternative live feeds can replace or at least supplement *Artemia* a preferred option would be to wean seahorses onto frozen diets as early as possible. Frozen diets may include purchased mysids, collected amphipods or even biofouling crustaceans which could be harvested from nets and frozen; however further work is required on extraction and grading of crustaceans. The ideal diet to replace *Artemia* in the culture of pot-bellied seahorses is an artificial diet that meets their requirements. More studies are therefore required in relation to artificial diets.

CHAPTER FOUR

HISTOLOGICAL DESCRIPTION OF THE DEVELOPMENT OF THE DIGESTIVE TRACT OF POT-BELLIED SEAHORSE JUVENILES

4.1 INTRODUCTION

During early development, fish larvae change from endogenous to exogenous feeding and for this to be successful larvae must be able to competently ingest and digest exogenous sources of food before the yolk sac and oil globule are depleted (Green & McCormick, 2001; Pena et al., 2003; Thorisson, 1994). However at first feeding the digestive tract is still largely undifferentiated; enzyme activity is low and the gut is generally a simple, straight tube which is short and as a consequence results in rapid evacuation of ingested food particles (De Silva & Anderson, 1995). The development time of the digestive tract varies from species to species and in the case of most fish the digestive tract is not fully developed until just before metamorphosis (into an early juvenile) when fish undergo a series of rapid changes in their morphology, sensory systems, locomotor ability and digestive and hormonal physiology (Gordon & Hecht, 2002; Kjorsvik et al., 1991; Walford & Lam, 1993). It is important to study the development of the digestive tract as such an understanding of the ontogeny of the digestive system and its functional capabilities will help identify limiting factors during larval rearing, improve feeding strategies by allowing synchronisation of developmental stages with appropriate feeding practices, aid in the identification of new alternative diets and reduce bottlenecks in the weaning process (Gisbert et al., 2004; Green & McCormick, 2001; Hamlin et al., 2000; Jones et al., 1993; Pena et al., 2003; Person-Le Ruyet et al., 1993; Sarasquete et al., 1995).

Digestive systems of fish vary with their diet and are composed of two functional units: the alimentary tract which assimilates food and is generally composed of a mouth, buccal cavity, pharynx, oesophagus, stomach and intestine that terminates at the anus and the extramural organs (i.e. liver, gall bladder, pancreas) which metabolically process organic and inorganic substances contained within the alimentary tract (Elliot & Bellwood, 2003; Green & McCormick, 2001; Loewe & Eckmann, 1988; Pena et al., 2003). The buccal cavity and pharynx may contain

teeth and a tongue and have gill rakers through which food particles are channelled towards the oesophagus. The mouth, buccal cavity and pharynx are composed of a mucosal and a submucosal layer that are separated by the lamina propria. The mucosa is comprised of striated squamous epithelium, which ranges in thickness and goblet cells (type A) which are specialised for secretion of muco-substances that may aid in lubrication, absorption, transport of macromolecules, enzymatic cofactors and general mucus secretion (Arellano et al., 2002; Gisbert et al., 2004; Pena et al., 2003; Unal et al., 2001). The submucosa is composed of loose connective tissue (squamous cells) in the oral cavity and in the pharynx it is structured in longitudinally arranged striated muscle bundles (Baglole et al., 1997; Gisbert et al., 2004; Hussaini, 1947; Rogers, 1997; Specian & Oliver, 1991; Vedugo, 1990).

The oesophagus begins in the pharynx and either joins to the stomach, or directly into the intestine depending on the species, via an oesophageal valve that ensures food is not regurgitated and is comprised of four layers (Pena et al., 2003). The mucosa rests on a thick basement membrane and is composed of stratified squamous epithelium with goblet cells (type A) in the pharynx. Proximal to where the oesophagus (caudally) enters the stomach the mucosal folds are longer, the epithelium cells gradually change from squamous to a columnar cell type and there are fewer goblet cells (Gisbert et al., 2004; Pena et al., 2003). The submucosa layer has a diverse cell population (arteries, veins, lymphocytes, fat cells) and is made up of a loose connective tissue layer separated from the mucosa by the stratum compactum layer, which replaces the lamina propria (Gisbert et al., 2004; Gordon & Hecht, 2002). The outer two layers include a wide muscularis and a thin serosa that are comprised of a circular stratified muscle, which is well vascularised, and connective tissue (simple squamous epithelium) respectively (Gisbert et al., 2004; Hussaini, 1947; Unal et al., 2001).

The stomach, if present, is the last organ of the digestive system to differentiate and can be simple and straight, u-shaped or y-shaped depending on the diet of the fish. Regardless of shape generally it is divided into three regions: the corpus ventriculi, pars pylorica and a gastric cecum (Gisbert et al., 2004). The fundic stomach (corpus ventriculi-gastric cecum region) and pyloric stomach (pars pylorica) show both primary and secondary longitudinal folding of the mucous membranes and are composed of four layers (mucosa, submucosa, muscularis, and serosa) (Loewe & Eckmann, 1988; Sarasquete et al., 1995). The stomach mucosa is comprised of simple ciliated columnar epithelial cells, simple tubular gastric glands (which increase in number with age) and a thin lamina propria which forms a network of loose connective tissue and supports the glands (Gordon & Hecht, 2002; Gisbert et al., 2004; Green & McCormick, 2001; Pena et al., 2003). The mucosa of the pyloric stomach differs from that of the fundic stomach in that there are no gastric glands within the lamina propria and the complexity of the mucosal folds decreases caudally to the pyloric sphincter, which separates the stomach from the anterior intestine (Gisbert et al., 2004; Sarasquete et al., 1995). The submucosa, which extends into the primary folds of the mucosa, is comprised of an outer layer of loose connective tissue and an inner stratum compactum of dense collagenous connective tissue. The muscularis layer is bilayered with an inner circular muscle layer, which is well vascularized, and is separated from the outer layer of longitudinal smooth muscle by the myenteric nerve plexus of Auerbach. The circular muscle layer is generally thicker than the longitudinal layer with the circular layer in carnivores, for example European seabass (*Dicentrarchus labrax*) being three times that of the longitudinal layer in the corpus ventriculi, ten times thicker in the gastric cecum and four to five times thicker in the pars pylorica. This bilayered muscularis arrangement and the serosa are evident throughout the digestive tract (Gisbert et al., 2004; Hussaini, 1947).

The anterior intestine and, if present, the pyloric ceca which originate immediately caudal to the pyloric sphincter are different from the stomach in that they have primary and secondary mucosal folds which are long and complex, the columnar epithelial cells have a more prominent brush border, type B goblet cells are present and the submucosa layer and muscular tissue layer are thinner (Gisbert et al., 2004; Gordon & Hecht, 2002; Green & McCormick, 2001; Pena et al., 2003; Unal et al., 2001). During development the intestine, which was originally a simple, undifferentiated straight tube, begins to loop around itself to accommodate the increasing length of the digestive tract in the abdominal cavity (Loewe & Eckmann, 1988; Pena et al., 2003). The wall of the rectum is similar in composition to the intestine except that the primary mucosal folds are shorter, there are fewer secondary folds and the primary mucosal folds become flattened near the anal pore (Gordon & Hecht, 2002; Pena et al., 2003; Unal et al., 2001). The intestine and rectum are separated by a valve, which consists of a circular connective tissue flap with a central opening that is directed caudally within the rectum lumen (Pena et al., 2003). The wall of the valve consists of both a mucosal and submucosal layer, which are continuous with those of the intestine, and only the circular layer of the intestinal muscularis is present in the valve (Green & McCormick, 2001; Loewe & Eckmann, 1988). The muscularis layer is different in the intestine and rectum with the longitudinal layer being wider than the circular layer in the rectum. Proximal to the anus, the circular muscle terminates and the longitudinal muscle and myenteric plexuses merge with the ventral body wall. The serosa of the intestine and rectum is continuous with that of the stomach and it is separated from the longitudinal muscle layer by a connective tissue layer, which caudally expands to surround the anus. The serosa becomes continuous with the lining of the peritoneal cavity (Gisbert et al., 2004; Hussaini, 1947; Unal et al., 2001).

The extramural organs include the liver, gall bladder and pancreas and can be absent at hatching but start to differentiate at 1 - 2 days after hatch. The liver is

positioned ventral to the oesophagus, is generally u-shaped and conforms to the viscera and peritoneal cavity. It is enclosed within a fibroconnective tissue capsule and features exocrine pancreatic tissue that surrounds portal veins and a bilaminar meshwork of hepatocytes (which appear as polyhedral cells with a centrally located nuclei) that comprises the basic functional hepatic units and accumulations of tissue macrophages (Green & McCormick, 2001; Pena et al., 2003; Sarasquete et al., 1995; Unal et al., 2001). The primary function of the liver is to assimilate nutritive substances, hematopoiesis, and destruction of red blood cells, and is by far the largest and most versatile of the metabolic organs.

The gall bladder is situated in the space between the liver and anterior intestine (fovea vesica bilaris) and is composed of the basic four layer structure (mucosa of columnar epithelium, a longitudinally folded submucosa of connective tissue, muscularis of smooth muscle, and a mesothelial serosa) found in the digestive tract (Unal et al., 2001). The gall bladder primarily functions as a storage receptacle for bile produced by the liver and the bile is delivered to the anterior intestine via the ductus choledochus, which is connected to the common bile or cystic duct.

There is exocrine and endocrine pancreatic tissue. Exocrine pancreatic tissue may surround portal veins within the liver and this pancreatic tissue extends from its origin beneath the serosa of the intestine. Endocrine pancreatic tissue occurs as a large principal islet and several smaller islets adjacent to the gall bladder (Gisbert et al., 2004). Pancreatic tissue is made up of two alveolar cell types; a columnar cell attached by its basal end to the portal veins and the typical pyramidal acinar cell, which in groups (five to eight) form lobular acini with central lumina (Green & McCormick, 2001; Pena et al., 2003; Sarasquete et al., 1995; Unal et al., 2001).

If culture of the pot-bellied seahorse is to be economically feasible, there is a great need to reduce their dependence on *Artemia.* To identify and implement new alternative diets an understanding of the ontogenetic development of the digestive system of seahorses is required. Currently there is little information available on the development of the digestive tract of seahorses thus this study aims to investigate the development of the digestive system of the pot-bellied seahorse, *Hippocampus abdominalis* from birth to 56 days old. The morphological and cellular development of the digestive tract in juvenile seahorses was examined using dissection and wax embedding/histological preparation and presented using description, diagrams and photographs. While chapter five (Ontogeny of the gut enzymes of pot-bellied seahorses) will provide information on the level and type of specific enzymes this chapter examines the possible sites of enzyme secretion by staining tissue sections for proteins.

4.2 Materials and Methods

A cohort of 100 newborn seahorses, from a single birth, were transferred from Seahorse World Pty. Ltd. to the Aquaculture Centre. Seahorses were kept in a 50 L natural coloured fibreglass (fawn coloured sides) conico-cylindrical tank with a white gelcoat base within a recirculation system. The tank was cleaned daily and the seahorses were fed *Artemia* enriched with Algamac™ at a feed rate of 5% body weight day^{-1}. The culture was maintained for 56 days.

From this population, five seahorses were collected for histological analysis at each time interval (day 0, 1, 5, 7, 14, 21, 35, 49, and 56). Prior to euthanasia (benzocaine overdose) and storage in Bouin's fixative, total length (tip of tail to tip of crown), head length, snout length and gape were measured (Figure 4.1) on individual fish by capturing images with a (Leica DC 300F) digital camera on a dissecting microscope (Olympus BH-2) and IM50 software and then using a measurement analysis package (Sigma Scan Pro 5). A small incision was made in the back of each seahorse to aid penetration of the fixative.

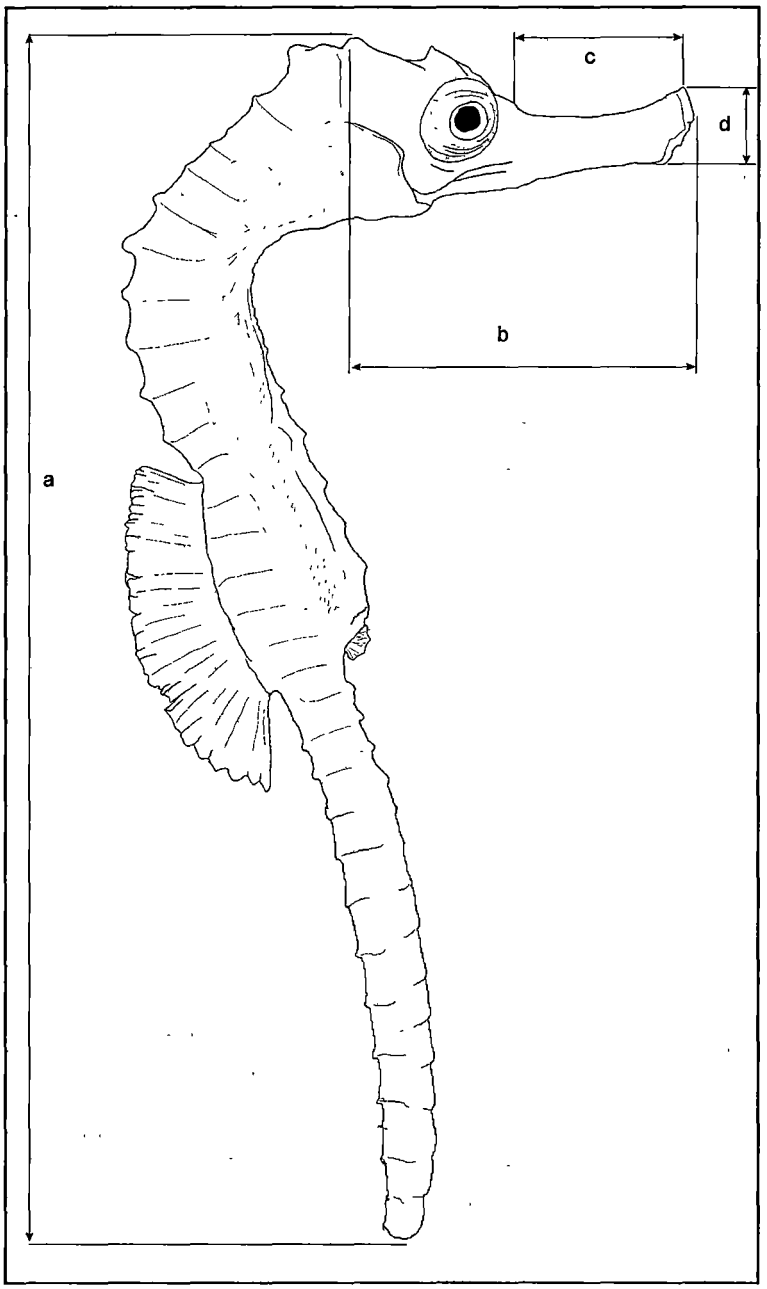


Figure 4.1. Drawing showing the morphological measurements of (a) total length, (b) head length, (c) snout length and (d) gape height.

Histology

Seahorses were removed from Bouin's fixative, washed with 70% ethanol and processed for wax histology in a Tissue-Tek II, 24 hour tissue processor. Seahorses, minus tail, were then embedded in paraffin wax and orientated to allow for both longitudinal and transverse sectioning. Older seahorses were cut into two, with the cut being made directly above the dorsal fin, as whole seahorses would not fit in the wax blocks. Blocks were serially sectioned (5µm) with a Microm HM 340 Microtome. Tissue sections were mounted on glass slides, dried for 24 hours and stained with Hematoxylin & Eosin to describe the development of the digestive tract and Mecuric Bromophenol Blue to highlight gut morphology or to elucidate the location of proteins (Chapman, 1975) respectively. Coverslips were then added using DPX.

Sections were examined using an Olympus BH-2 microscope and images of the digestive tract and areas of protein were captured with a Leica DC 300F digital camera and IM50 (LEICA IM50) software. Images were stored as .jpg files and transferred through Paint Shop Pro 5 into this chapter. No features were erased from the images and colour was not adjusted. Images were adjusted for brightness and focus using the drawing palette in Microsoft Word 2000. Colour plates were printed with an Epson Stylus PHOTO 830 printer.

4.3. RESULTS

On release from the pouch pot-bellied seahorses on average weighed 0.0094 ± 0.002 g and had a total length of 16.68 ± 1.47 mm and at 56 days of age seahorses weighed 0.22 ± 0.023 g and had a total length of 53.6 ± 1.44 mm (Table 4.1).

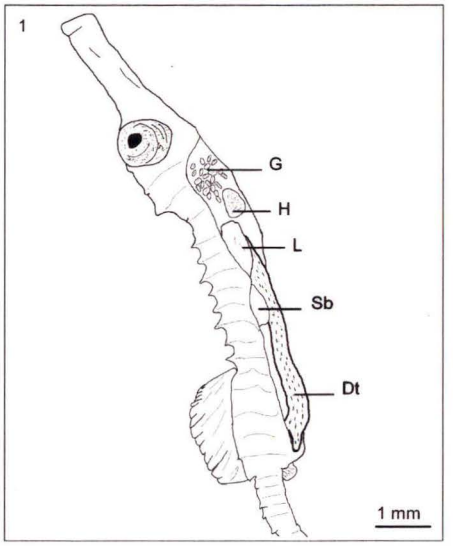
4.3.1. GENERAL ANATOMY

A seahorse body is supported by an internal skeleton (skull, vertebral column and fin rays) of cartilage and ossified calcium and their major organs include the heart, liver, gall bladder, swim bladder, pancreas, kidney and intestine (Figure 4.2 - 4.5). The heart, liver, and gall bladder are found in the neck region of the seahorse. The kidney runs along the backbone, the swim bladder is found along the upper most part of the backbone while the exocrine pancreatic tissue is found within the liver and endocrine pancreatic tissue is located at the base of the intestine near the anus. The digestive tract of seahorses includes a buccal cavity which does not possess teeth or a tongue, an oesophagus that enters directly into the intestine, an intestine which coils with age and does not appear to be separated into sections (top, mid and lower) and a rectum that is separated from the intestine by an intestinal valve. Seahorses do not develop a stomach (Figure 4.6 - 4.23).

Table 4.1. The weight, total length, head length, snout length and gape height of different aged pot-bellied seahorses from the same cohort.

Age (days)	Weight (g)	Total length (mm ± SE)	Head length (mm ± SE)	Snout length (mm ± SE)	Gape height (mm ± SE)
0	0.009 ± 0.002	16.7 ± 1.47	4.38 ± 0.29	2.34 ± 0.12	1.08 ± 0.03
7	0.013 ± 0.001	20.2 ± 0.73	4.38 ± 0.21	2.52 ± 0.24	1.11 ± 0.01
14	0.027 ± 0.003	25.0 ± 1.01	6.06 ± 0.34	3.32 ± 0.27	1.41 ± 0.05
21	0.041 ± 0.003	30.0 ± 1.30	7.02 ± 0.29	4.24 ± 0.03	1.46 ± 0.03
35	0.118 ± 0.007	42.2 ± 1.16	8.80 ± 0.12	4.92 ± 0.09	1.76 ± 0.06
49	0.191 ± 0.015	50.4 ± 1.57	11.16 ± 0.29	6.26 ± 0.13	2.01 ± 0.02
56	0.224 ± 0.023	53.6 ± 1.44	11.36 ± 0.31	6.02 ± 0.23	2.09 ± 0.06

Figure 4.2. Scientific drawing and a photograph of a dissected newborn pot-bellied seahorse. (1) Scientific drawing showing the layout of the internal organs of a seahorse, (2) Photograph showing the layout of the digestive tract Legend: Dt, digestive tract; G, gill; H, heart; L, liver; Sb, swim bladder.


1
G
H
L
Sb
Dt
1 mm

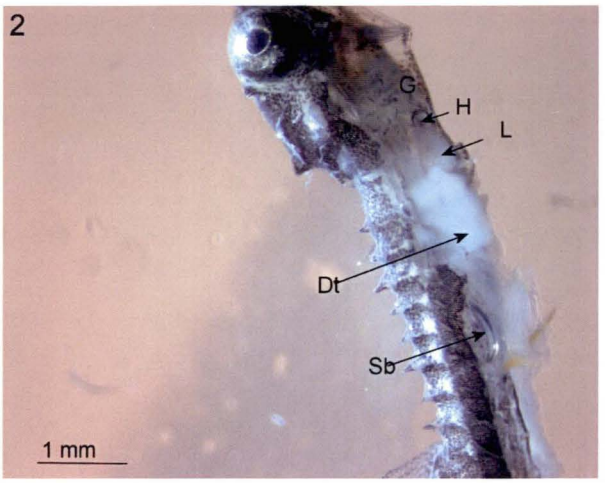
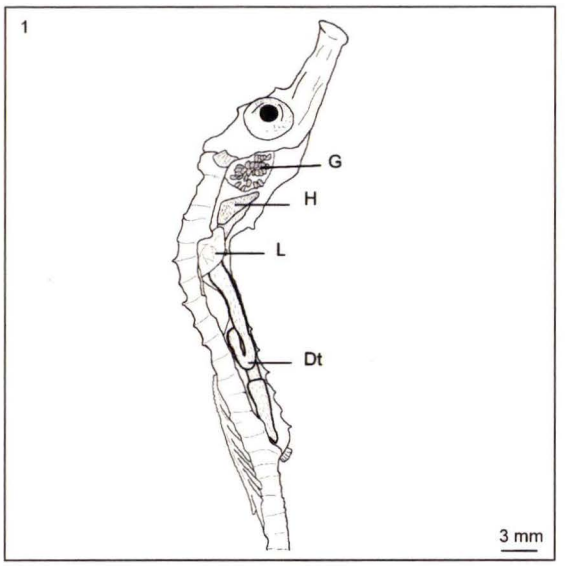
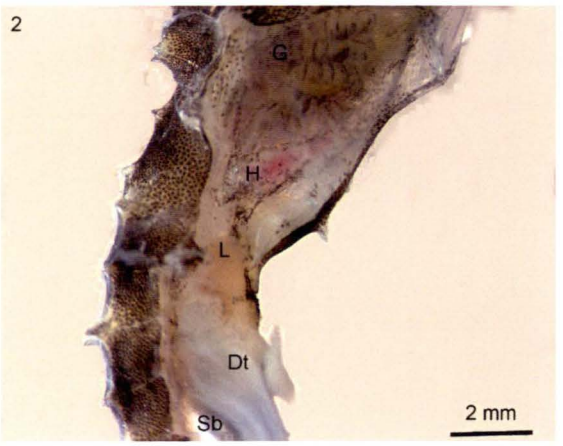

2
G
H
L
Dt
Sb
1 mm

Figure 4.3. Scientific drawing and photographs of dissected 1 month old seahorse. (1) Scientific drawing showing the layout of the internal organs. (2) Photograph showing the gills, heart, liver, swim bladder and top region of the digestive tract. (3) Photograph showing how the digestive tract loops in a one month old seahorse. Legend: Dt, digestive tract; G, gill; H, heart; L, liver; Sb, swim bladder.


1
G
H
L
Dt
3 mm


2
G
H
L
Dt
Sb
2 mm

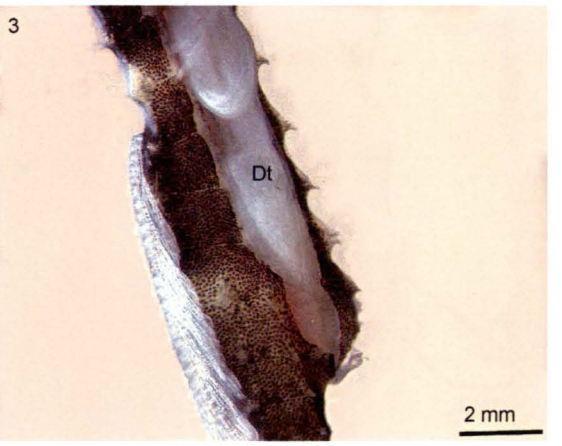
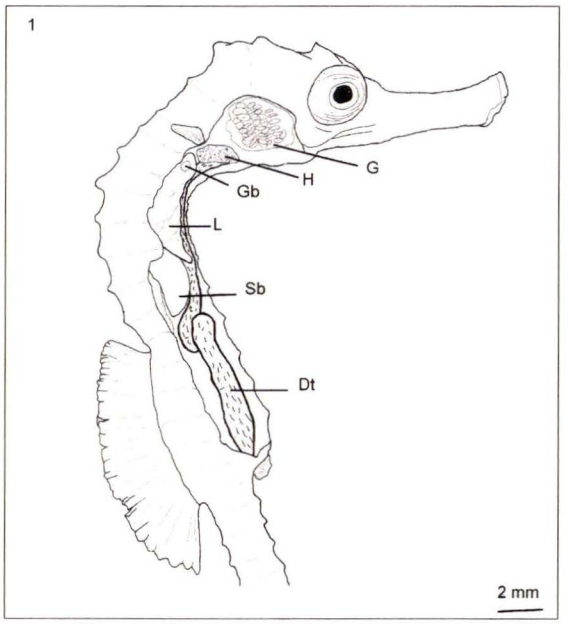
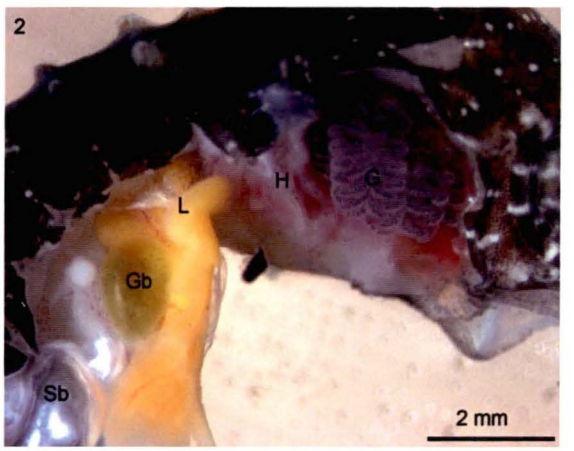
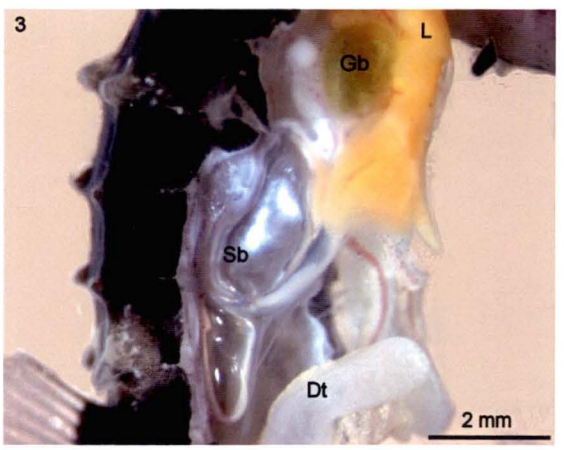

3
Dt
2 mm

Figure 4.4. Scientific drawing and photographs of a dissected 2 month old seahorse. (1) Scientific drawing showing the layout of the internal organs of a 2 month old seahorse. (2) Photograph displaying the gills, liver, gall bladder and top region of the digestive tract. (3) Photograph displaying the liver, swim bladder and loops in the digestive tract. (4) Photograph displaying the lower region of the digestive tract leading to the anus. Legend: Dt, digestive tract; G, gill; Gb, gall bladder; H, heart; L, liver; Sb, swim bladder.


1
G
H
Gb
L
Sb
Dt
2 mm


2
H
G
L
Gb
Sb
2 mm


3
L
Gb
Sb
Dt
2 mm

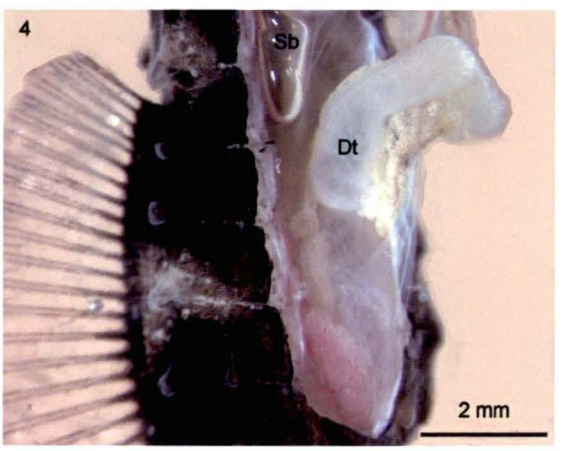
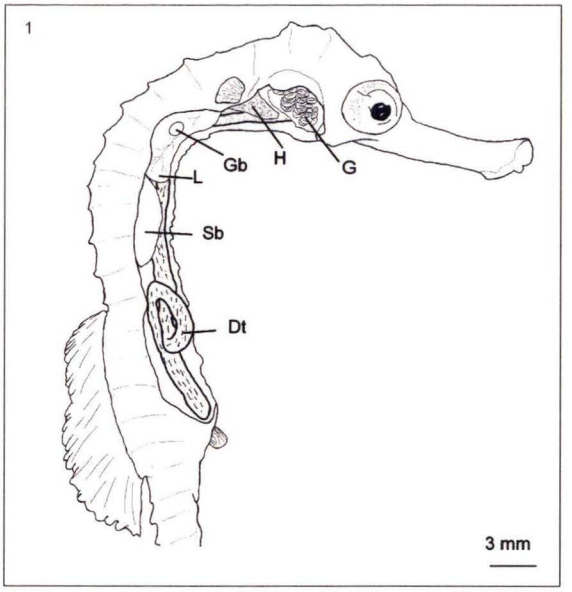
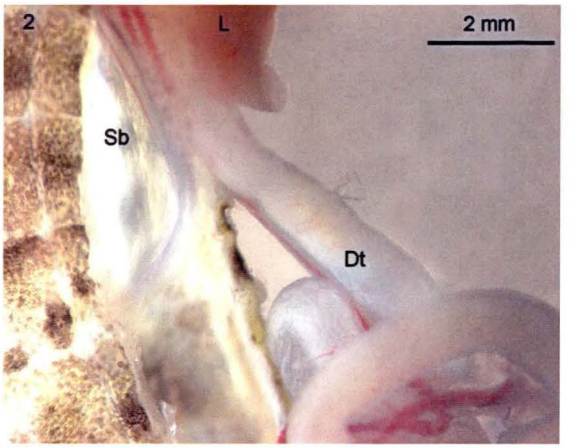
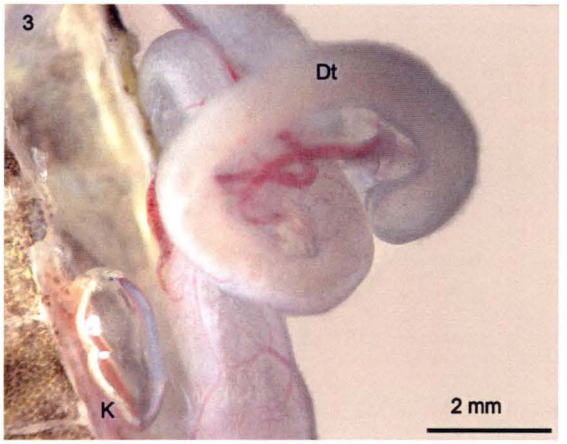
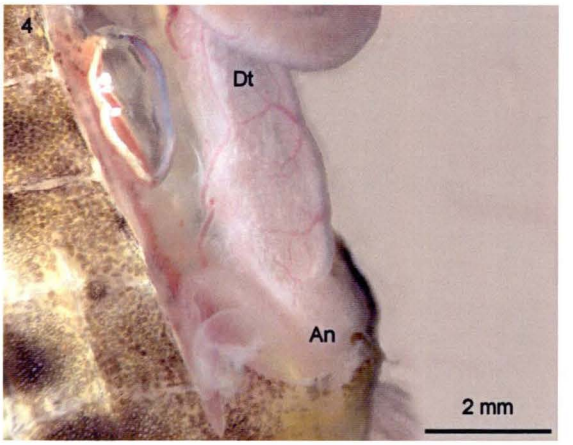

4
Sb
Dt
2 mm

Figure 4.5. Scientific drawing and photographs of a 3 month old seahorse. (1) Scientific drawing demonstrating the layout of the internal organs and the development of the digestive tract. (2) Photograph displaying liver, swim bladder and middle region of the digestive tract showing the loops in the gut. (3) Photograph displaying the loops in the digestive tract. (4) Photograph displaying the lower region of the digestive tract leading to the anus. Legend: Dt, digestive tract; G, gill; Gb, gall bladder; H, heart; L, liver; Sb, swim bladder.


1
Gb
H
G
L
Sb
Dt
3 mm


2
L
2 mm
Sb
Dt


3
Dt
K
2 mm


4
Dt
An
2 mm

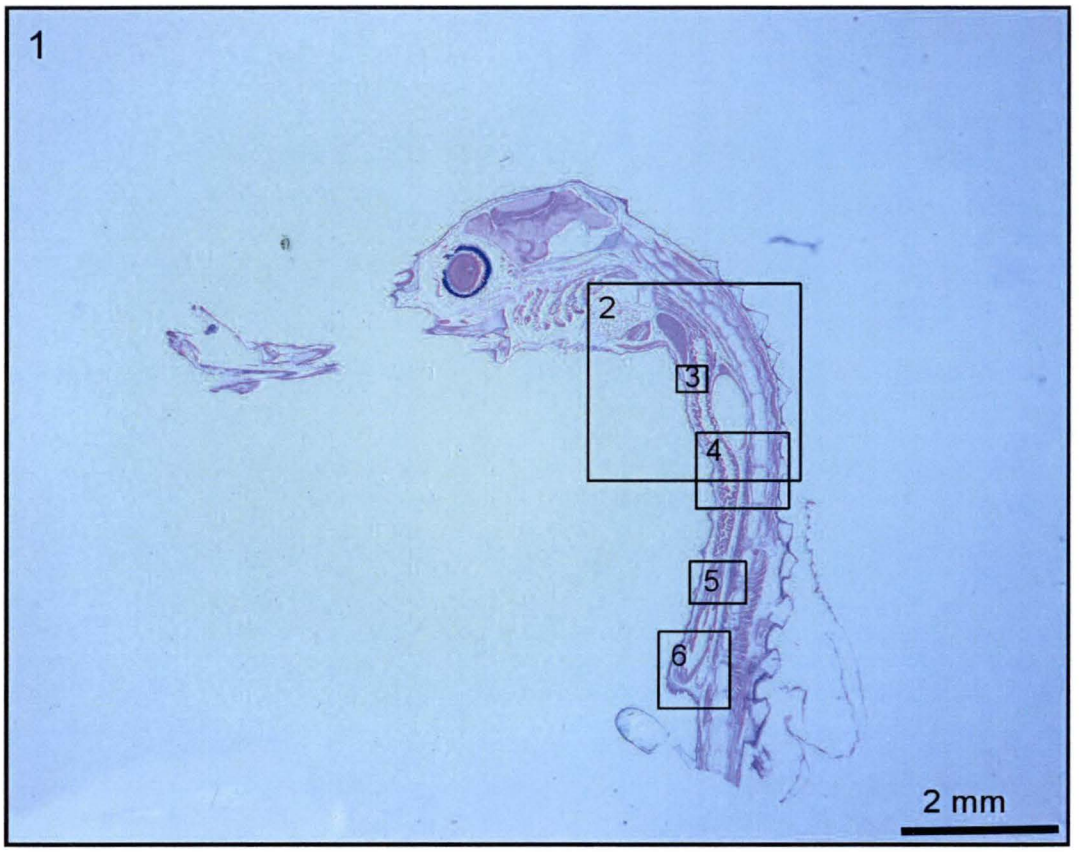
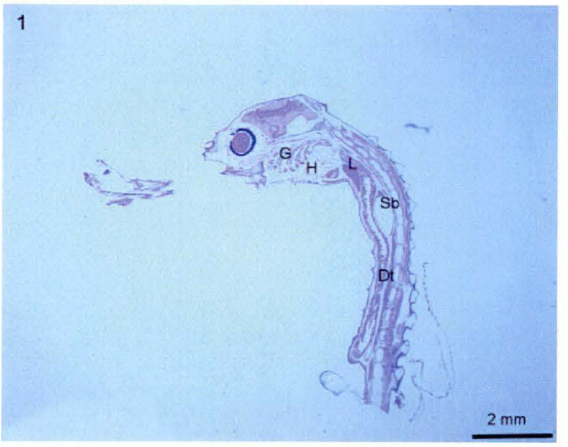
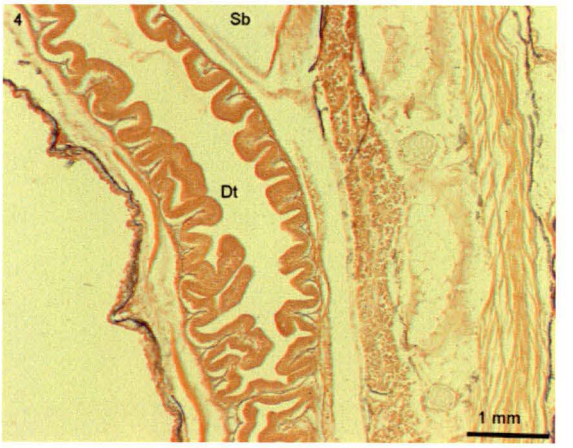


Figure 4.6. Approximate position of the longitudinal sections of the newborn seahorse stained with Haemotoxylin and Eosin. (1) The layout of the internal organs of a seahorse. (2) The liver, digestive tract and swim bladder of a newborn seahorse. (3) The top region of the digestive tract. (4) The middle region of the digestive tract. (5) The middle region of the digestive tract. (6) LS showing the lower gut and anus of a newborn seahorse. Legend: (An) anus; (Dt) digestive tract; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.

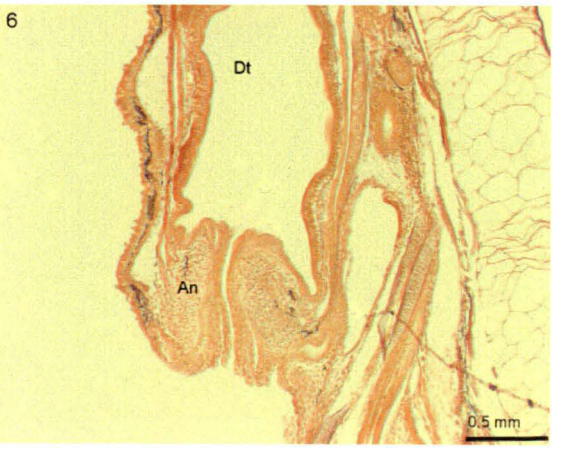

1
G
H
L
Sb
Dt
2 mm


2
L
H
Sb
Dt
2 mm


3
Dt
0.5 mm


4
Sb
Dt
1 mm


5
Dt
0.5 mm


6
Dt
An
0.5 mm

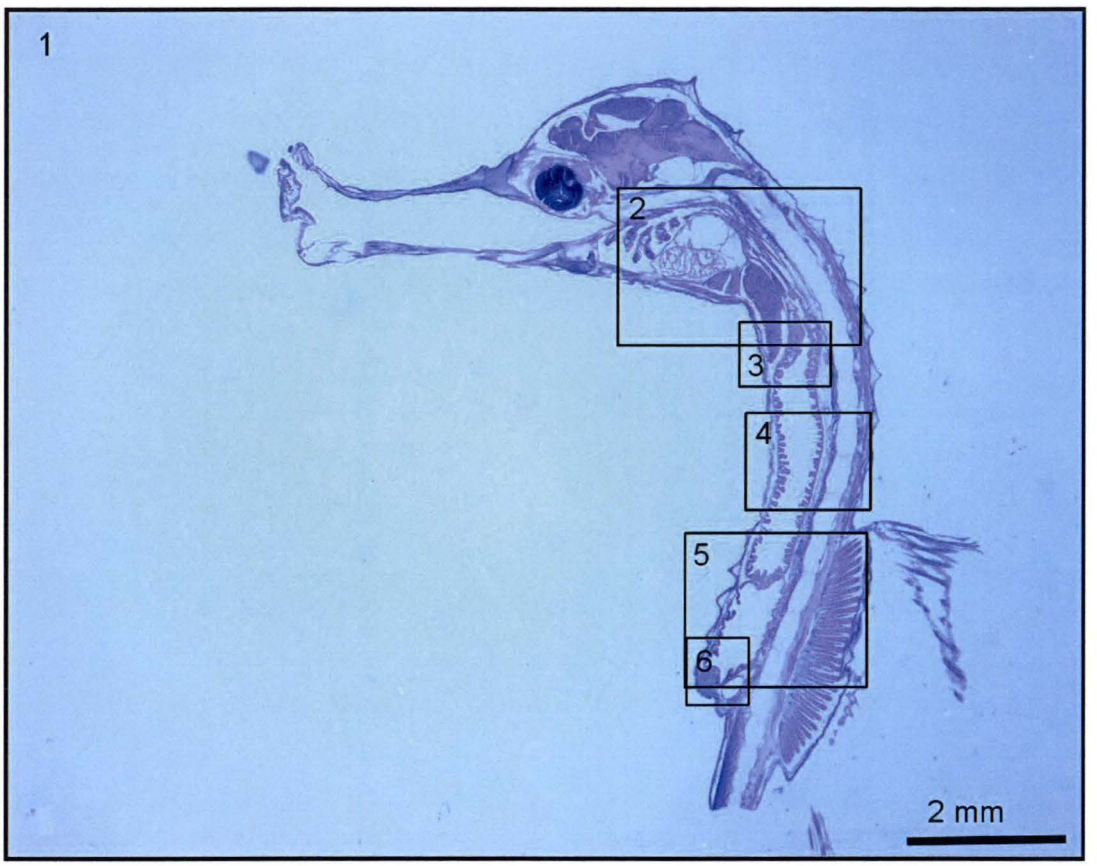
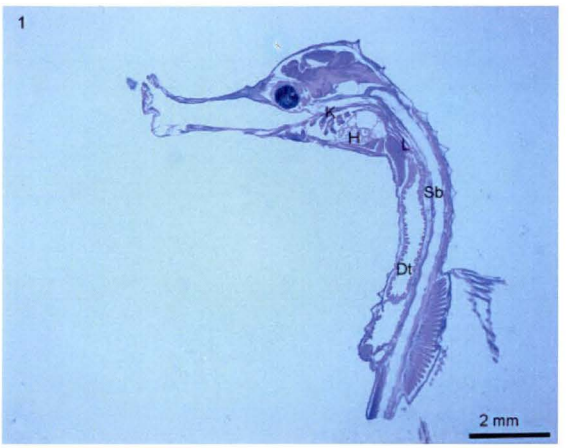
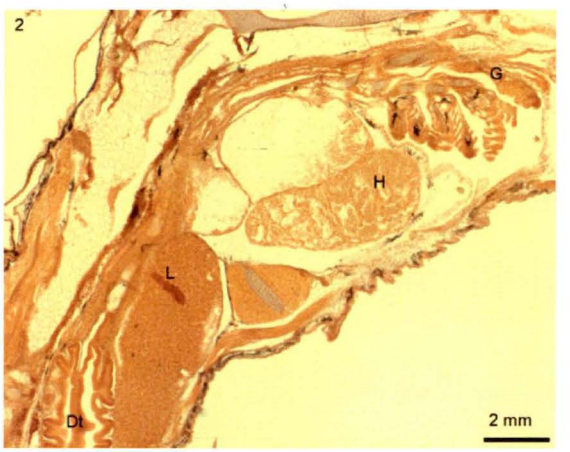


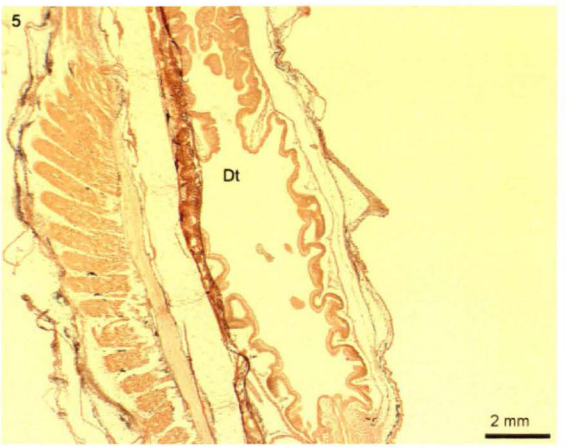
Figure 4.7. Approximate location of the longitudinal sections of the one day old seahorse stained with Haemotoxylin and Eosin. (1) The layout of the internal organs of a seahorse. (2) The liver and top region of the digestive tract. (3) The top region of the digestive tract and lower region of the kidney. (4) The middle region of the digestive tract. (5) The lower region of the digestive tract and the anus. (6) The anus of a seahorse. Legend: (An) anus; (Dt) digestive tract; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.

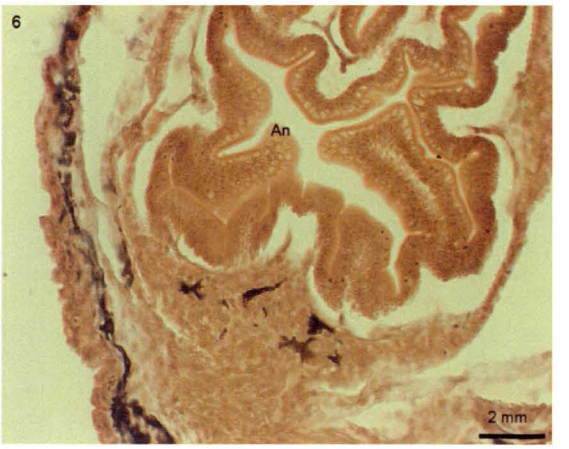

1
K
H
L
Sb
Dt
2 mm


2
G
H
L
Dt
2 mm


3
L
Dt
1 mm


4
L
Dt
2 mm


5
Dt
2 mm


6
An
2 mm

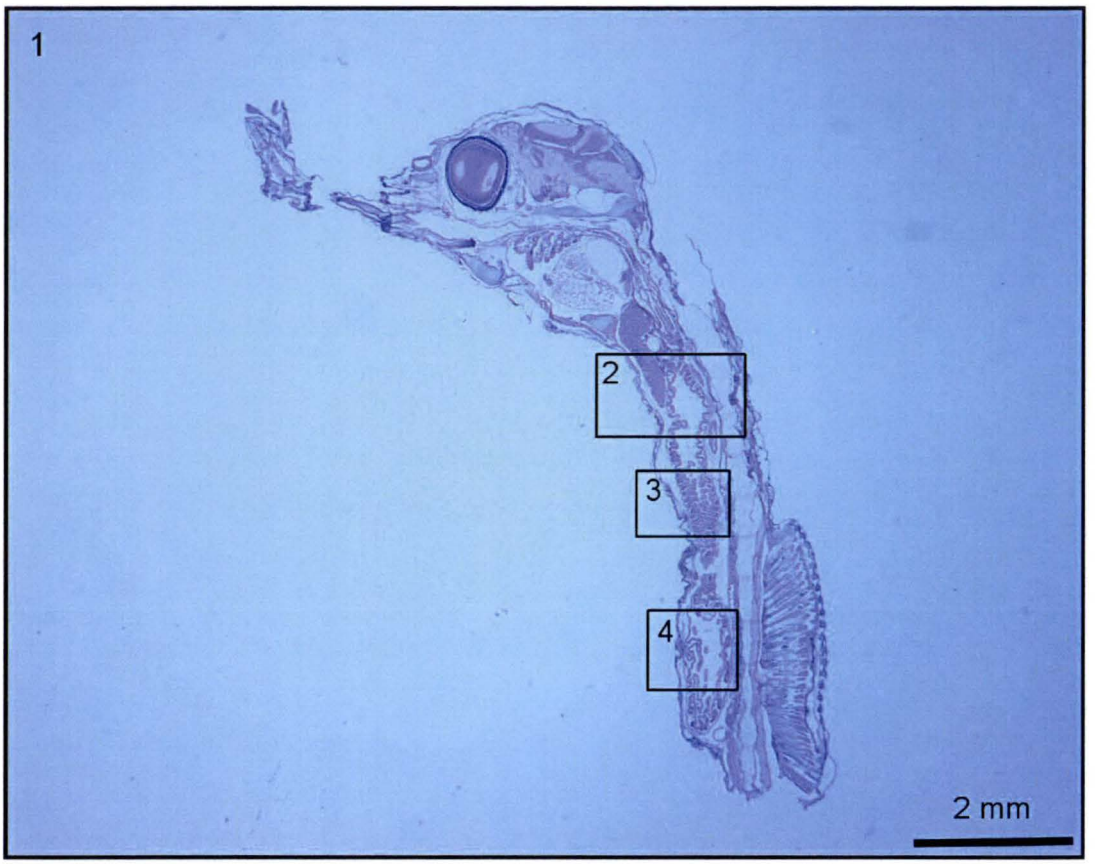
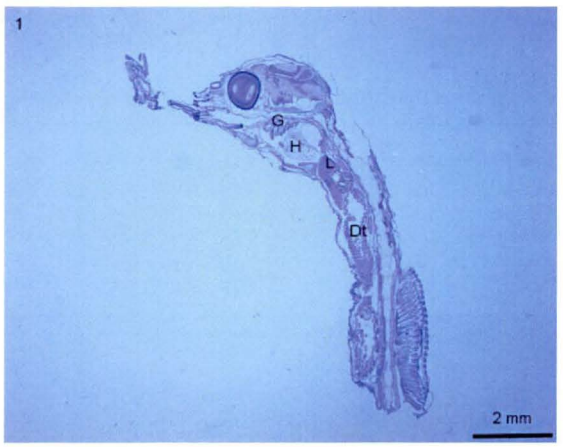
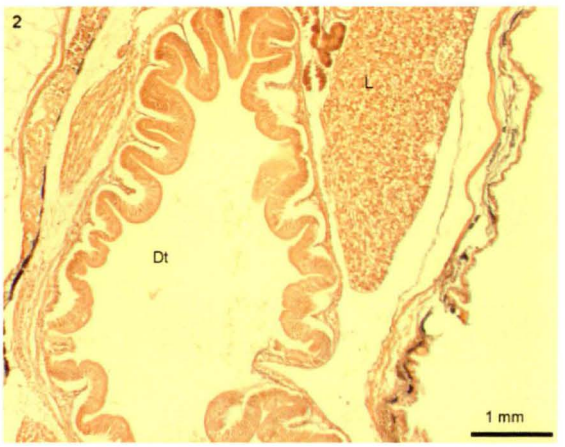
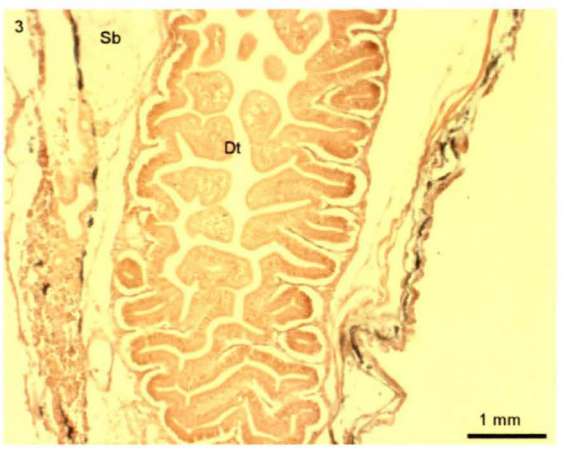
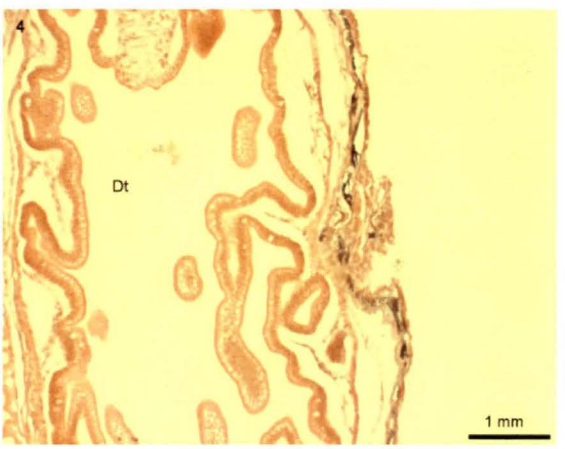


Figure 4.8. Approximate position of the longitudinal sections of the five day old seahorses stained with Haemotoxylin and Eosin. (1) The layout of the internal organs. (2) The top region of the digestive tract. (3) The end of the swim bladder and the middle region of the digestive tract. (4) The end region of the digestive tract leading to the anus. Legend: (Dt) digestive tract; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.


1
G
H
L
Dt
2 mm


2
L
Dt
1 mm


3
Sb
Dt
1 mm


4
Dt
1 mm

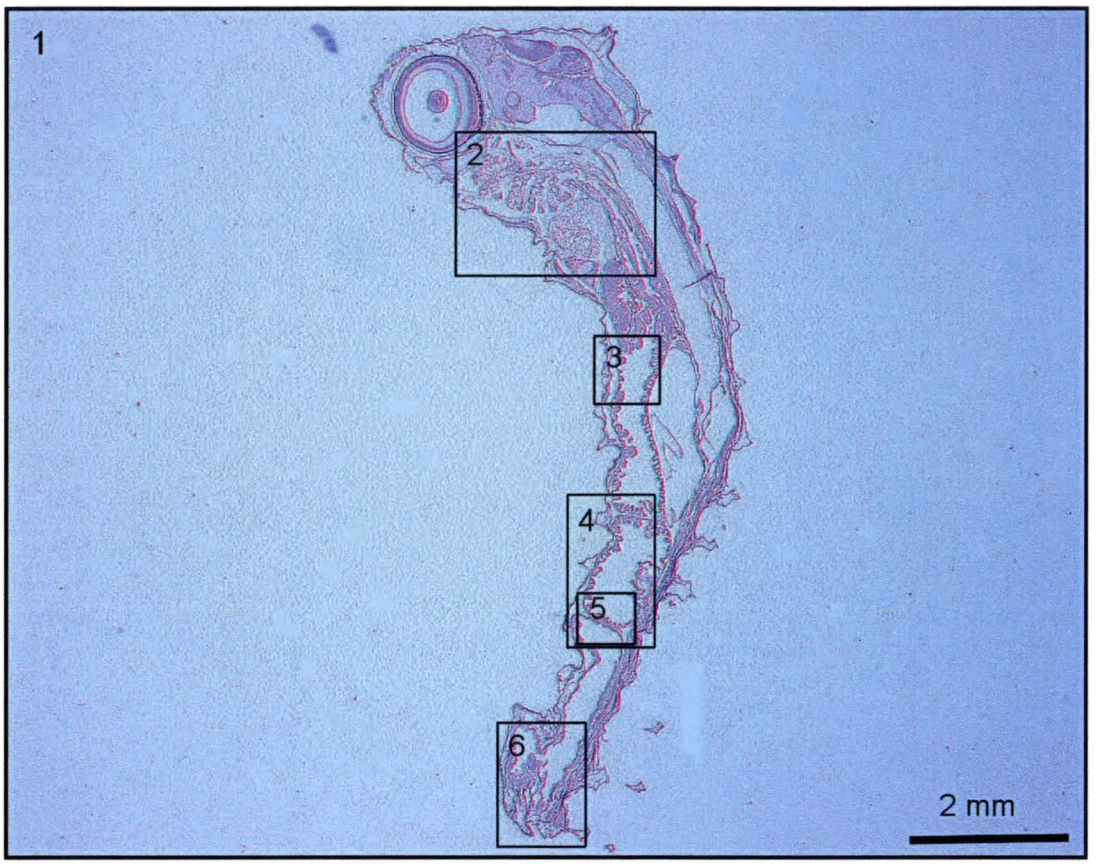
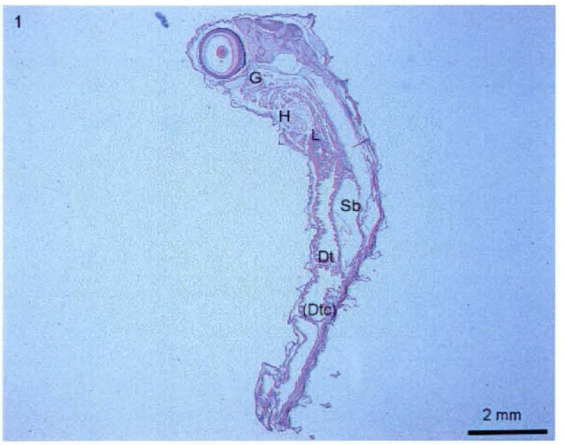
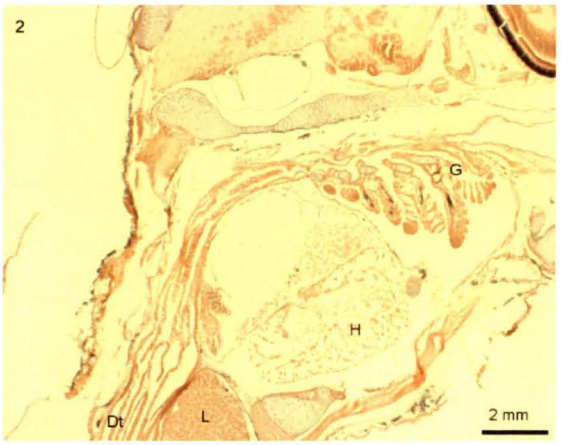
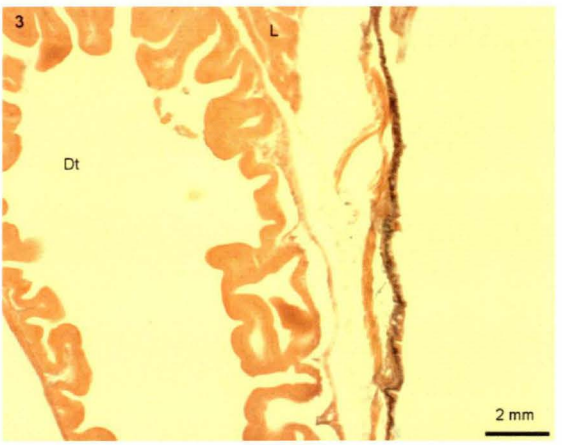
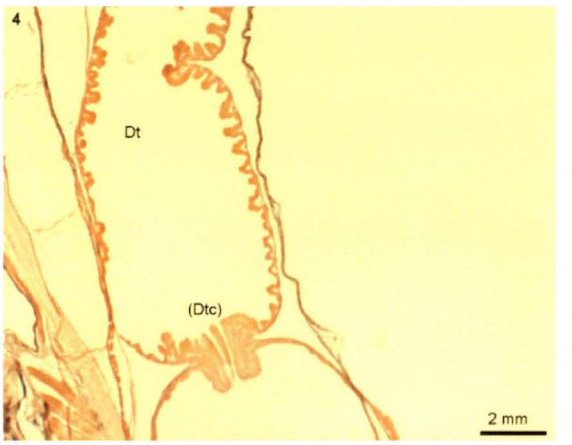


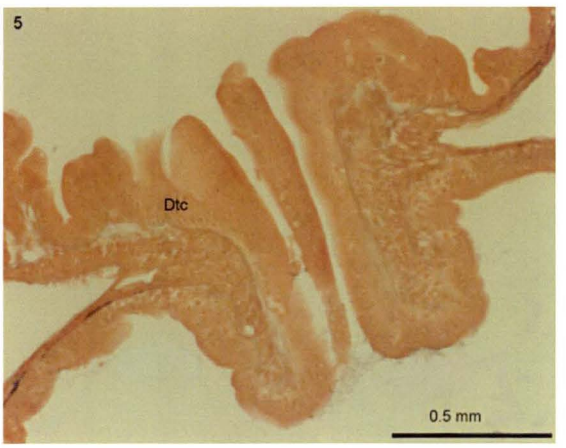
Figure 4.9. Approximate location of the longitudinal sections of a seven day old seahorse stained with Haemotoxylin and Eosin. (1) LS of the layout of internal organs of a seahorse. (2) The gills, heart, liver and top area of the digestive tract. (3) LS demonstrating the top region of the digestive tract. (4) The middle region of the digestive tract and the digestive tract constriction. (5) The constriction in the digestive tract. (6) The lower region of the digestive tract and the anus. Legend: (An) anus; (Dt) digestive tract; (Dtc) digestive tract constriction; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.

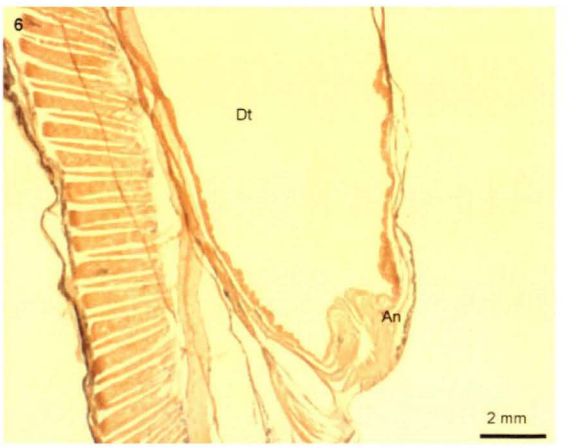

1
G
H
L
Sb
Dt
(Dtc)
2 mm


2
G
H
Dt
L
2 mm


3
L
Dt
2 mm


4
Dt
(Dtc)
2 mm


5
Dtc
0.5 mm


6
Dt
An
2 mm

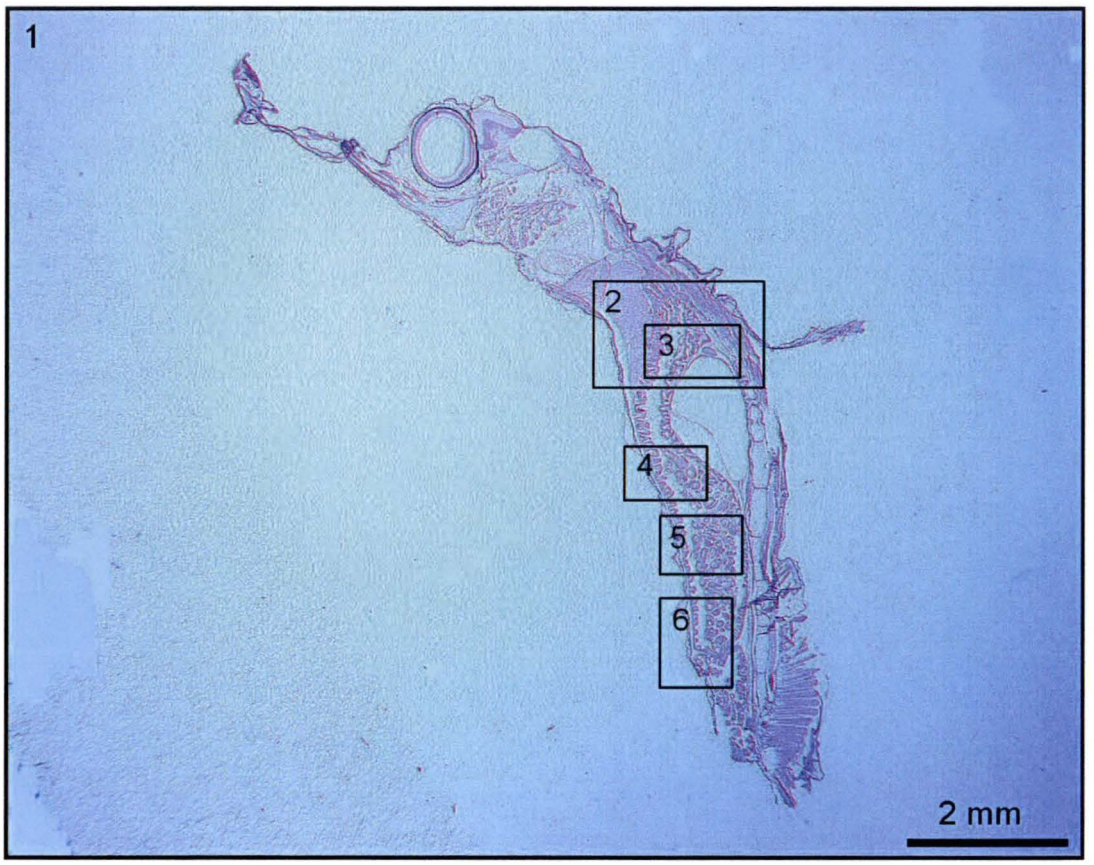
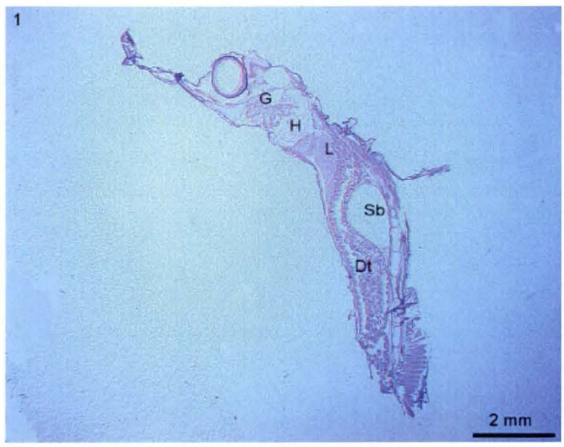
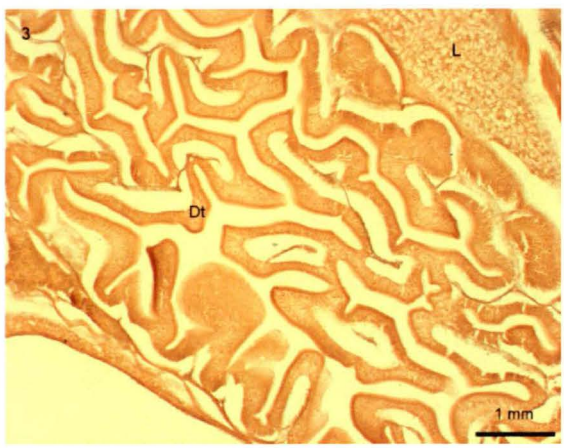
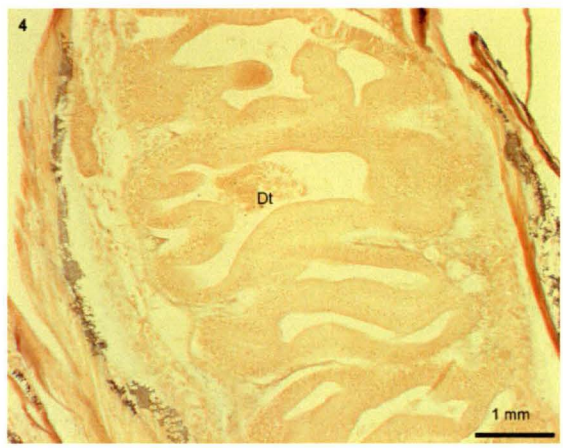


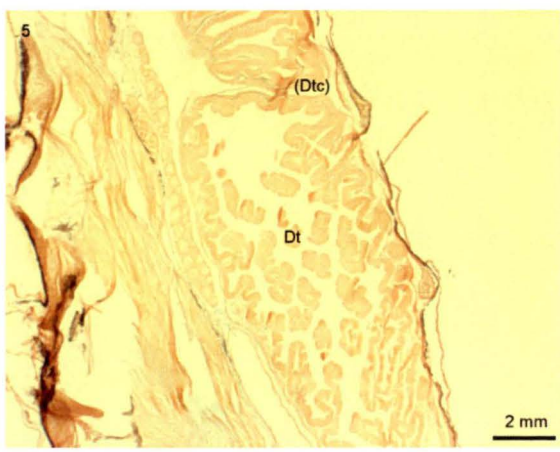
Figure 4.10. Approximate location of the longitudinal sections of a 14 day old seahorse stained with Haemotoxylin and Eosin. (1) The layout of the internal organs of a seahorse. (2) The liver, swim bladder and top region of the digestive tract. (3) LS demonstrating the top region of the digestive tract. (4) The middle region of the digestive tract. (5) The middle and lower region of the digestive tract and the constriction in the digestive tract. (6) The lower region of the digestive tract and the anus. Legend: (An) anus; (Dt) digestive tract; (Dtc) digestive tract constriction; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.

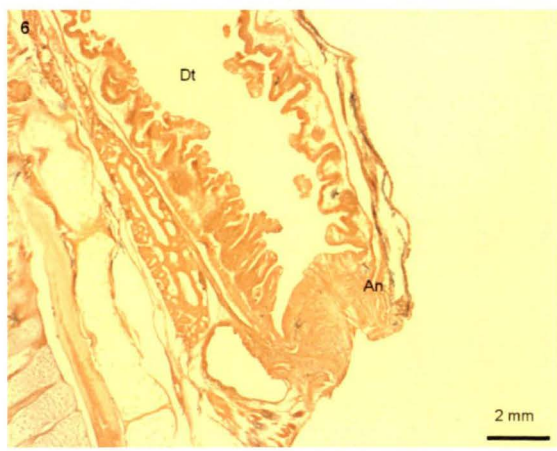

1
G
H
L
Sb
Dt
2 mm


2
L
Dt
Sb
2 mm


3
L
Dt
1 mm


4
Dt
1 mm


5
(Dtc)
Dt
2 mm


6
Dt
An
2 mm

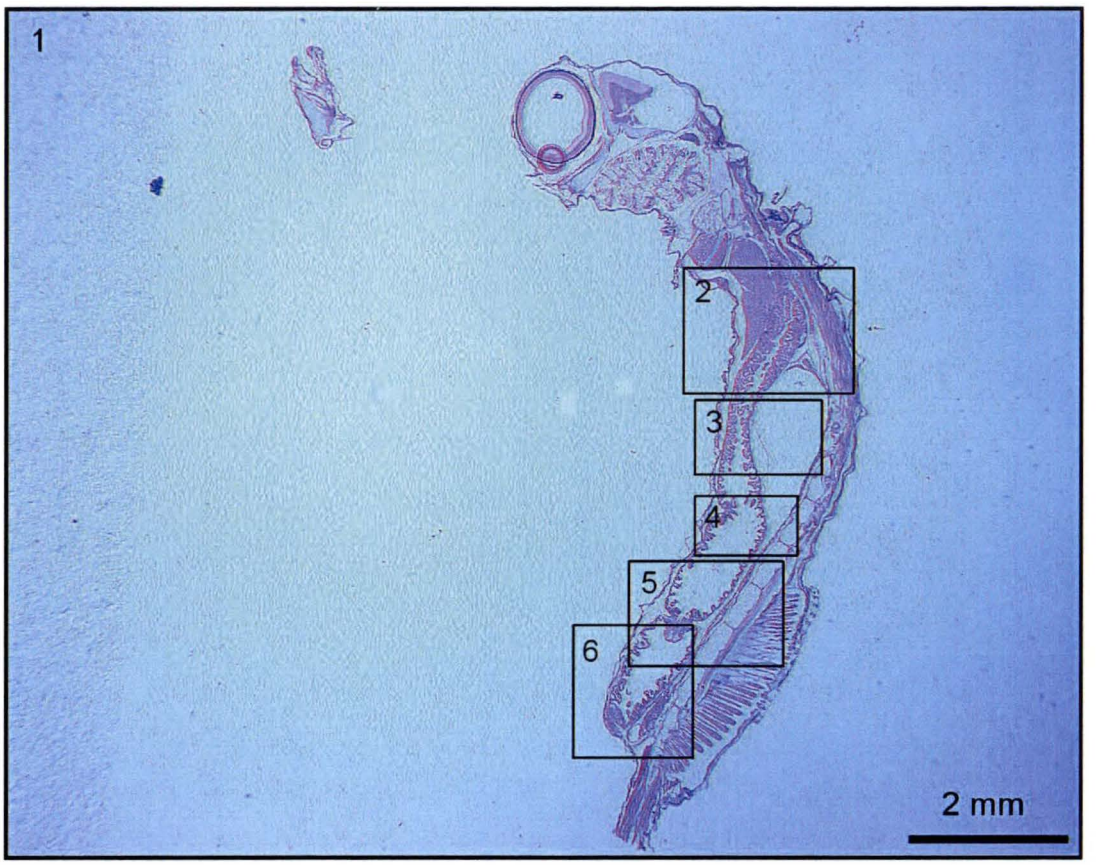
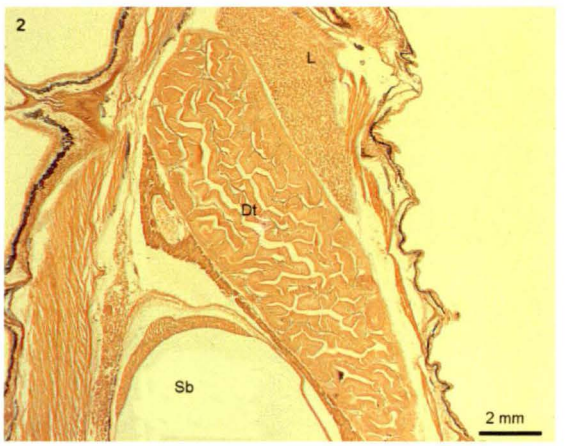
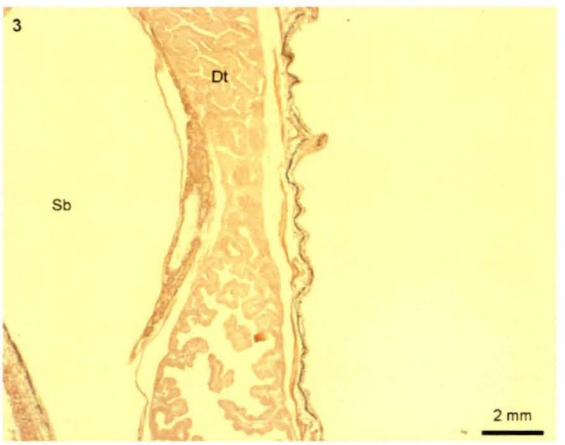
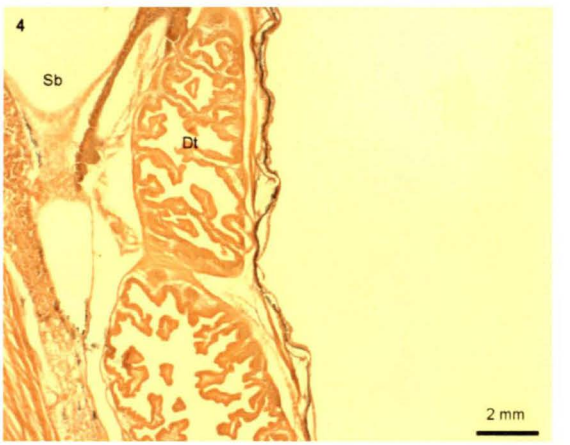


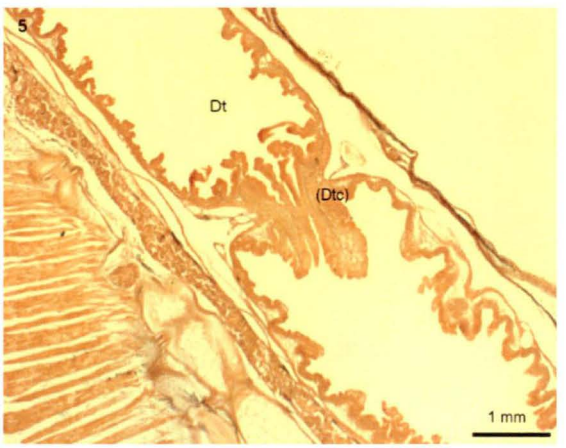
Figure 4.11. Approximate location of the longitudinal sections of 21 day old seahorses stained with Haemotoxylin and Eosin. (1) The layout of internal organs of a 21 day old seahorse. (2) The liver, top region of the digestive tract and swim bladder. (3) The swim bladder and middle region of the digestive tract. (4) The middle region of the digestive tract and the end of the swim bladder. (5) The constriction in the digestive tract and the lower region of the digestive tract. (6) The lower region of the digestive tract and the anus. Legend: (An) anus; (Dt) digestive tract; (Dtc) digestive tract constriction; (G) gill; (H) heart; (L) liver; (Sb) swim bladder.

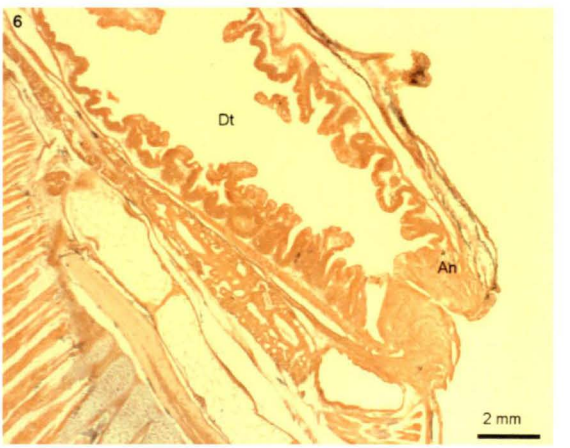

1
G
H
L
Sb
Dt
2 mm


2
L
Dt
Sb
2 mm


3
Dt
Sb
2 mm


4
Sb
Dt
2 mm


5
Dt
(Dtc)
1 mm


6
Dt
An
2 mm

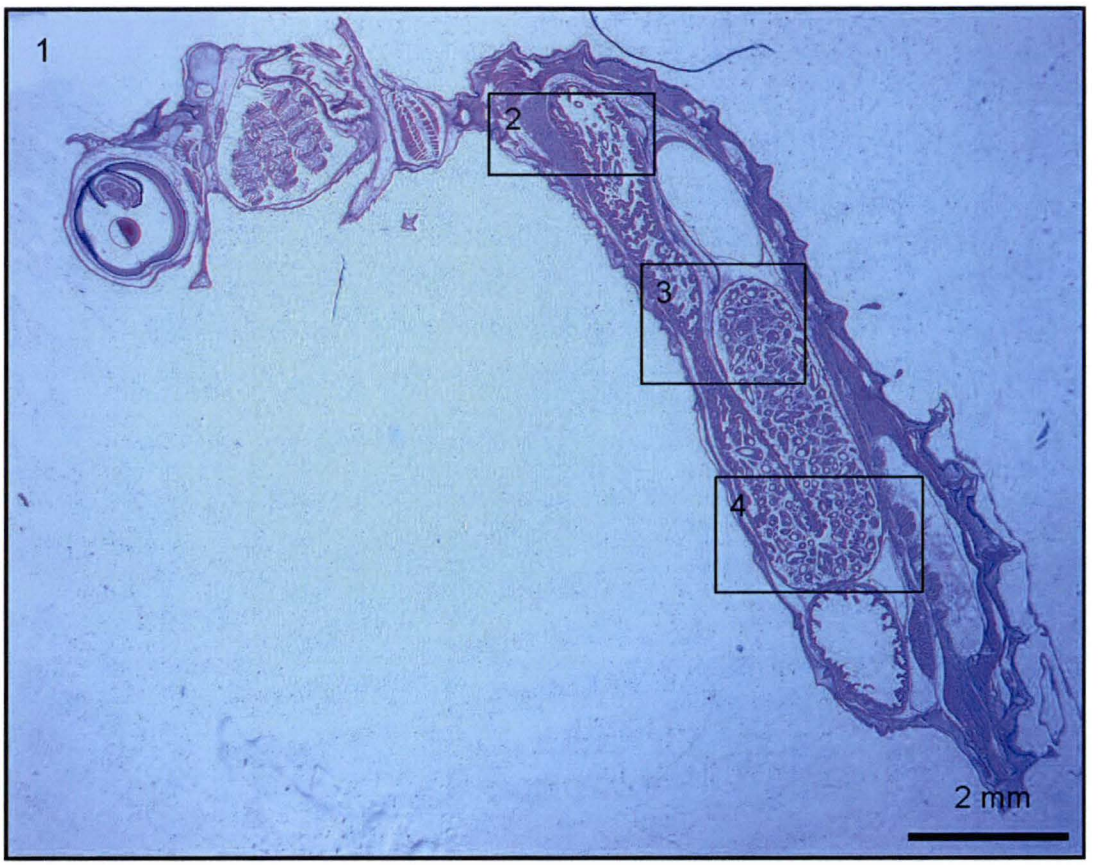
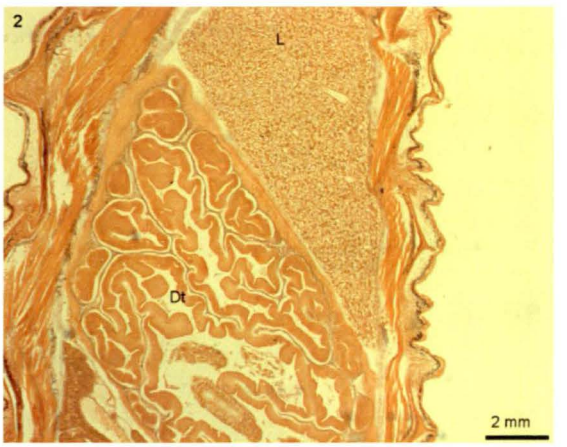
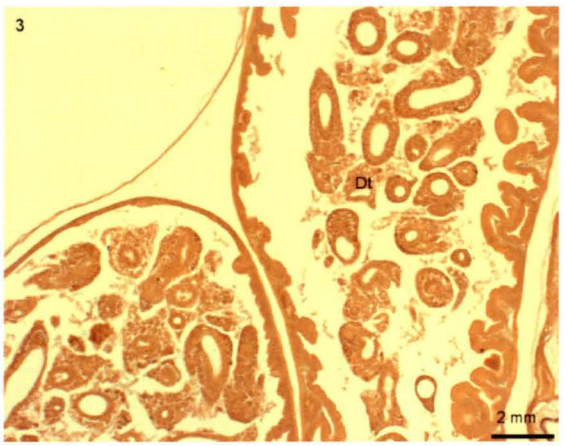
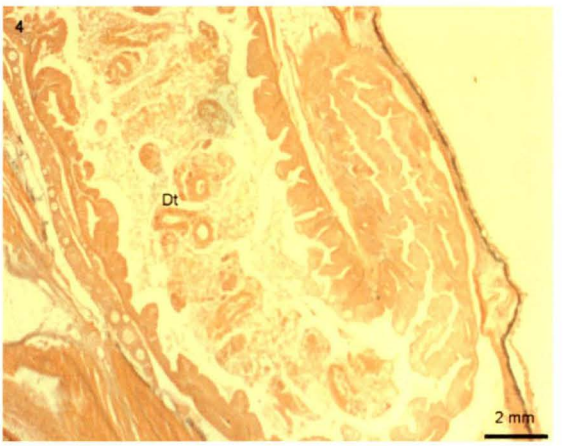


Figure 4.12. Approximate location of the longitudinal sections of a 35 day old seahorse stained with Haemotoxylin and Eosin. (1) The layout of internal organs of a 35 day old seahorse. (2) The liver and the top region of the digestive tract. (3) The middle region of the digestive tract and the loops in the gut. (4) The loops in the lower region of the digestive tract. Legend: (Dt) digestive tract; (G) gill; (L) liver; (Sb) swim bladder.


1
G
L
Sb
Dt
2 mm


2
L
Dt
2 mm


3
Dt
2 mm


4
Dt
2 mm

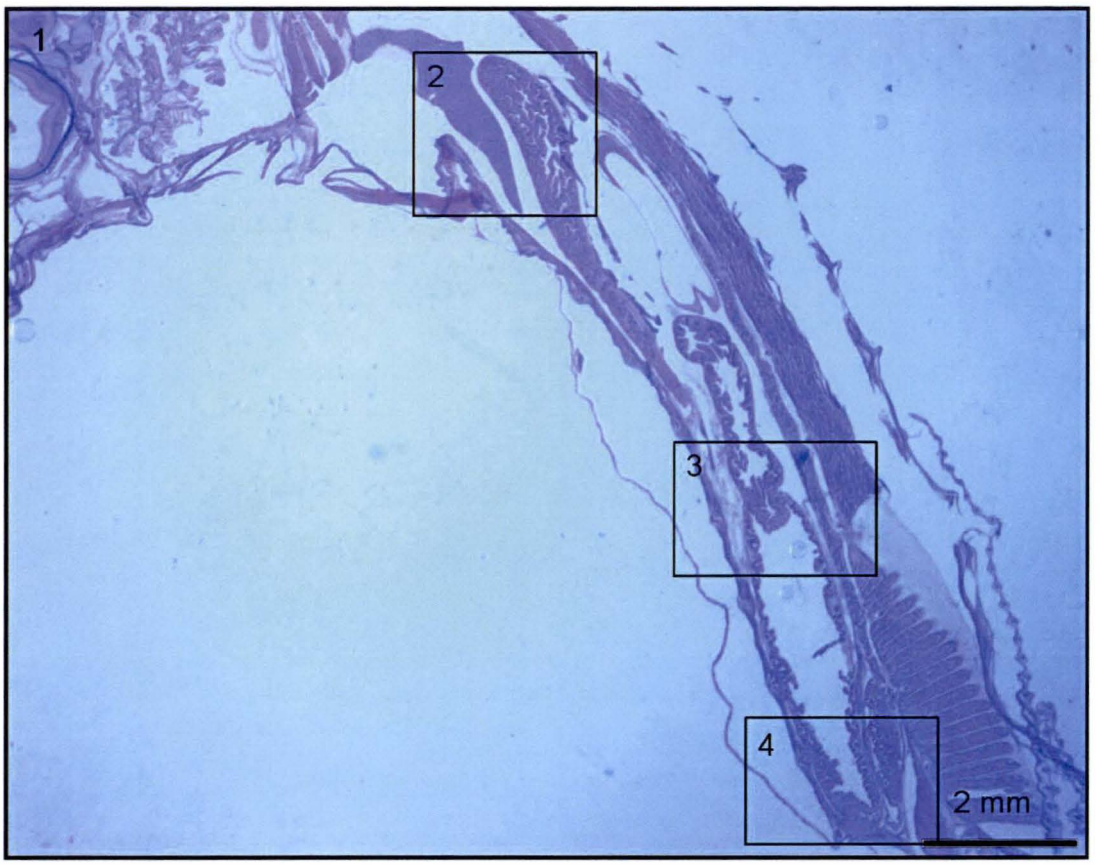
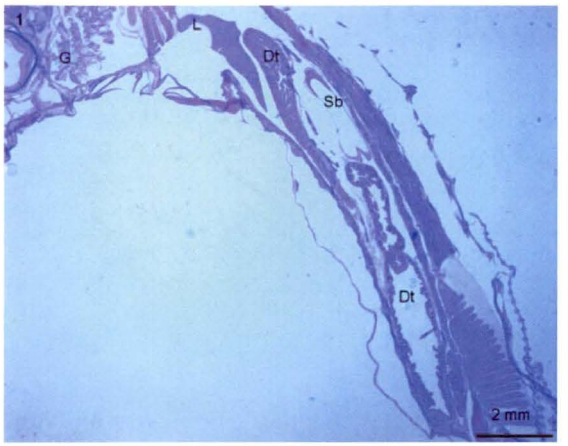
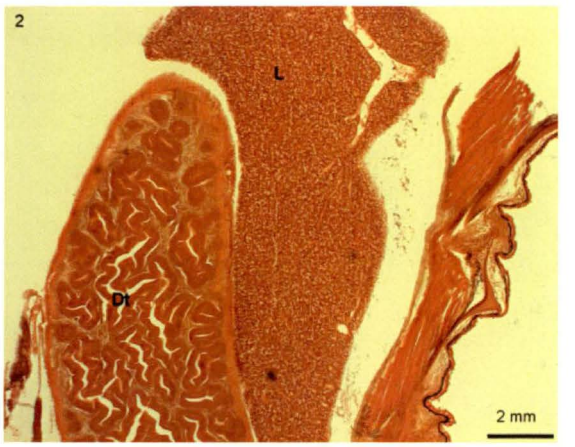
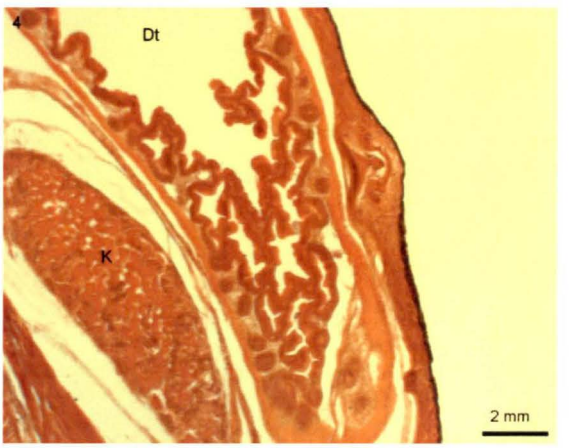


Figure 4.13. Approximate location of the longitudinal sections of a 49 day old seahorse stained with Haemotoxylin and Eosin. (1) The layout of internal organs of a seahorse. (2) The liver and top region of the digestive tract. (3) The middle and lower regions of the digestive tract and the constriction in the digestive tract. (5) The lower region of the digestive tract leading to the anus. Legend: (Dt) digestive tract; (Dtc) digestive tract constriction; (G) gill; (K) kidney; (L) liver; (Sb) swim bladder.


1
G
L
Dt
Sb
Dt
2 mm


2
L
Dt
2 mm


3
Dt
(Dtc)
2 mm


4
Dt
K
2 mm

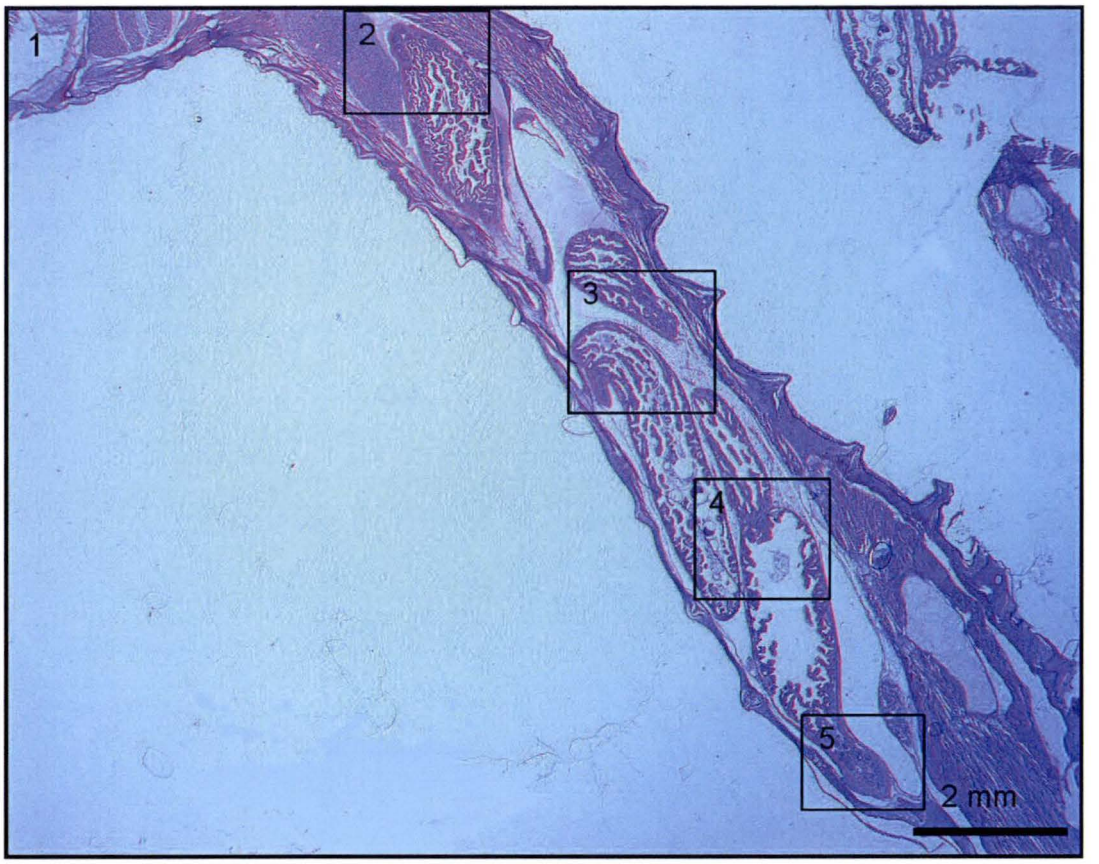
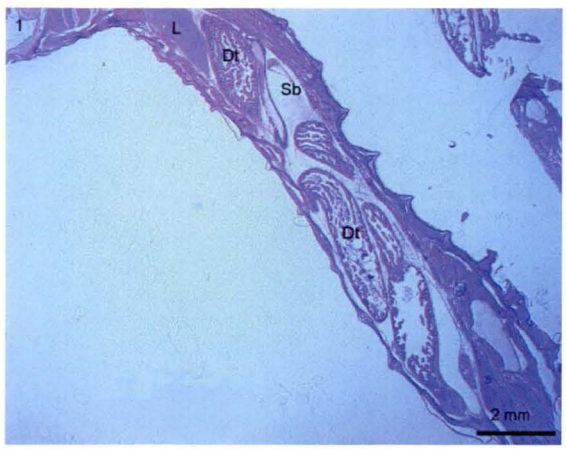
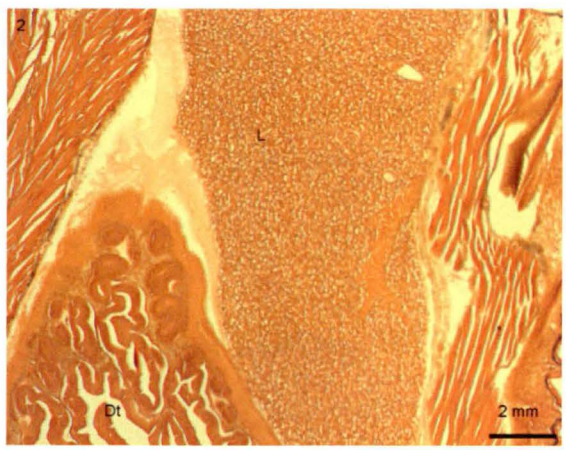
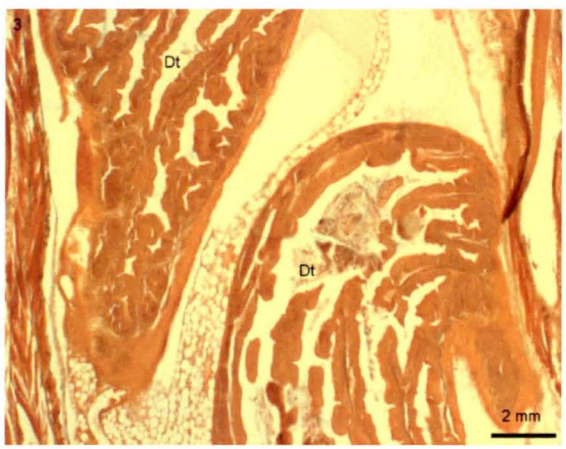
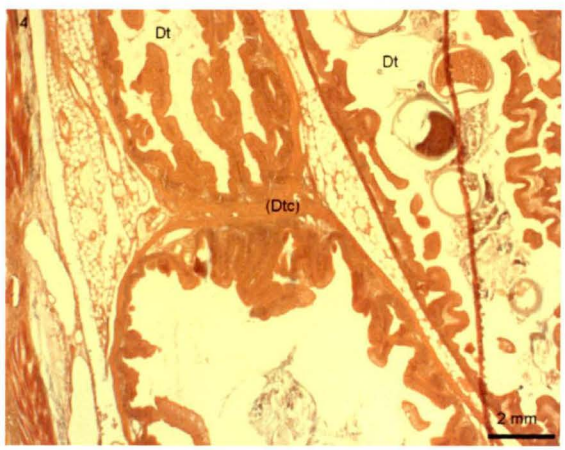


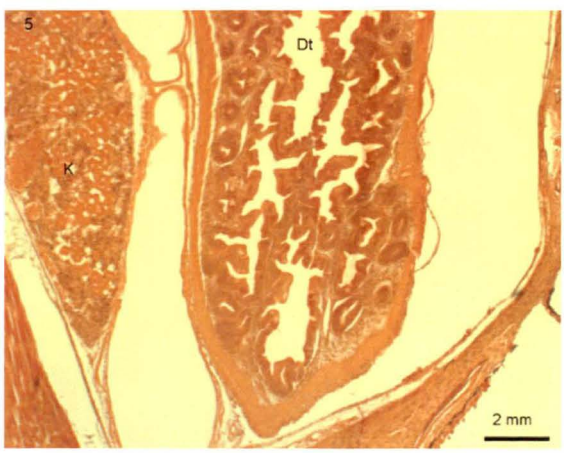
Figure 4.14. Approximate location of the longitudinal sections of a 56 day old seahorse stained with Haemotoxylin and Eosin. (1) The layout of internal organs of a seahorse. (2) The liver and top region of the digestive tract. (3) The middle region of the digestive tract showing how the gut loops. (4) The middle and lower regions of the digestive tract and the constriction in the digestive tract. (5) The lower region of the digestive tract leading to the anus. Legend: (Dt) digestive tract; (Dtc) digestive tract constriction; (K) kidney; (L) liver; (Sb) swim bladder


1
L
Dt
Sb
Dt
2 mm


2
L
Dt
2 mm


3
Dt
Dt
2 mm


4
Dt
Dt
(Dtc)
2 mm


5
Dt
K
2 mm

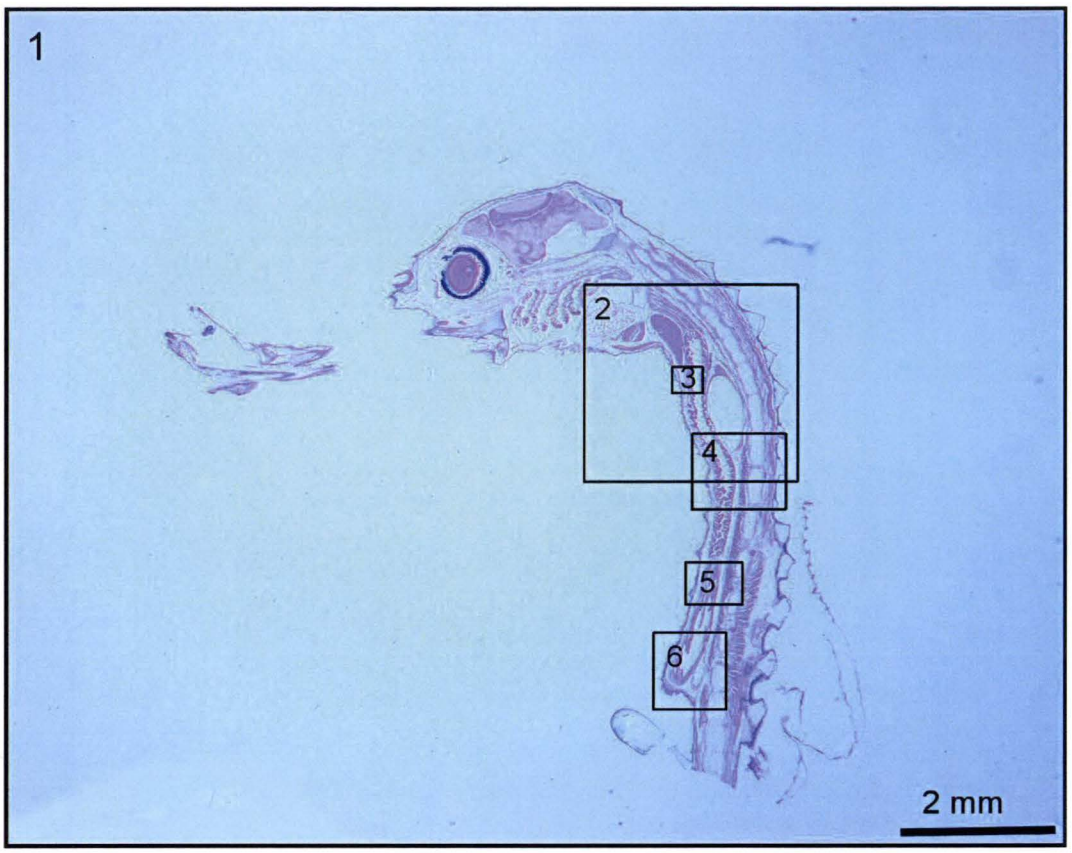
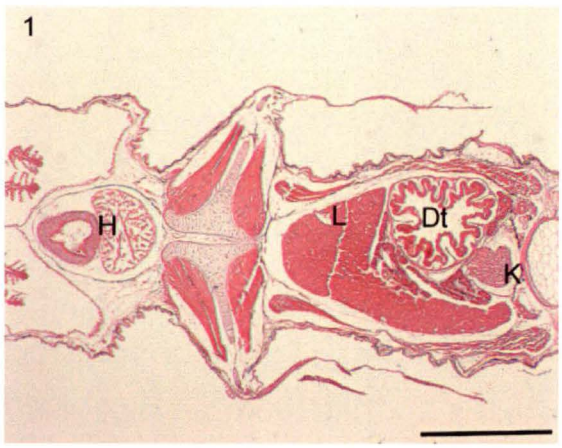
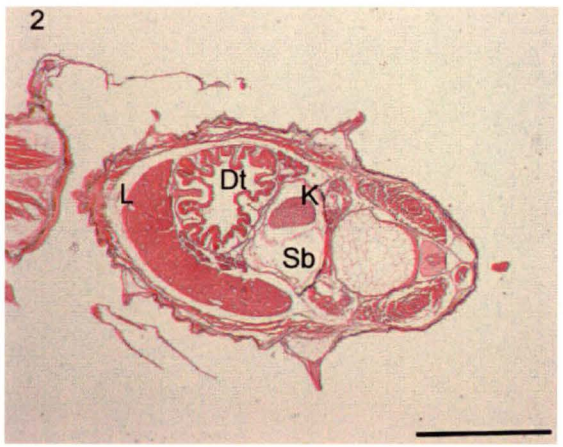
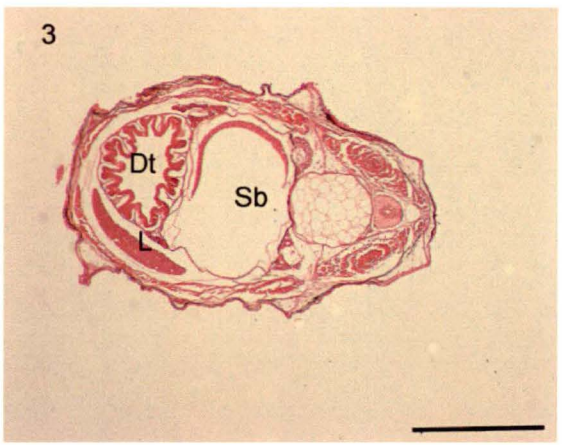
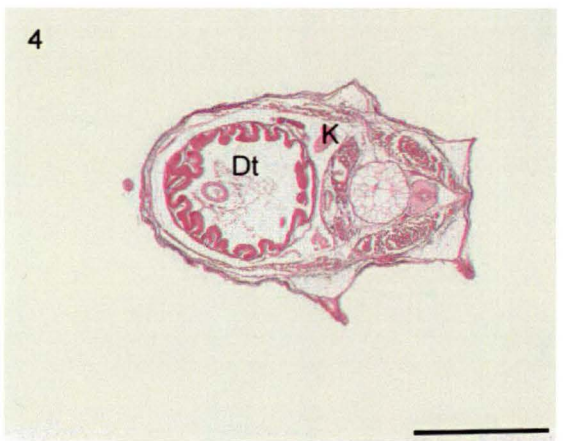


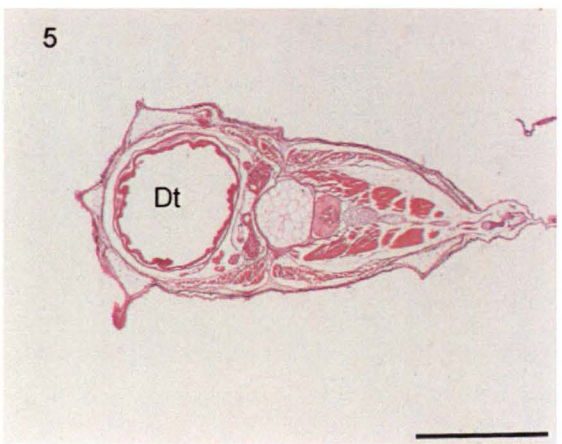
Figure 4.15. Approximate location of the transverse sections through a newborn seahorse stained with Haemotoxylin and Eosin. (1) The heart, liver and top region of the digestive tract (scale, 50 μm). (2) The liver, top of the swim bladder, digestive tract and kidney (scale, 50 μm). (3) The bottom of the liver, swim bladder enlarging, and the digestive tract (scale, 50 μm). (4) The digestive tract and kidney below the swim bladder (scale, 50 μm). (5) The lower region of the digestive tract (scale, 50 μm). (6) The lower digestive tract and anus (scale, 50 μm). Legend: An, anus; Dt, digestive tract; H, heart; K, kidney; L, liver; Sb, swim bladder.

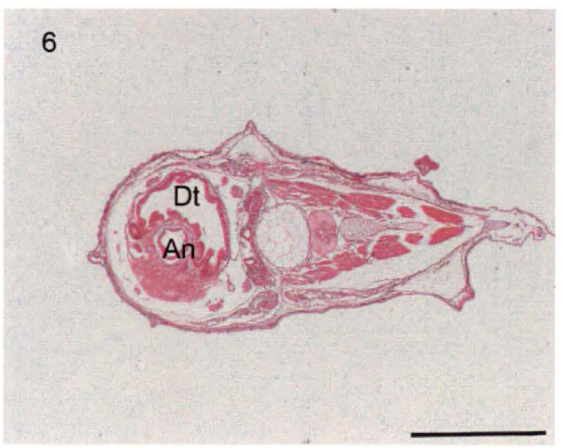

1
H
L
Dt
K


2
L
Dt
K
Sb


3
Dt
Sb
L


4
K
Dt


5
Dt


6
Dt
An

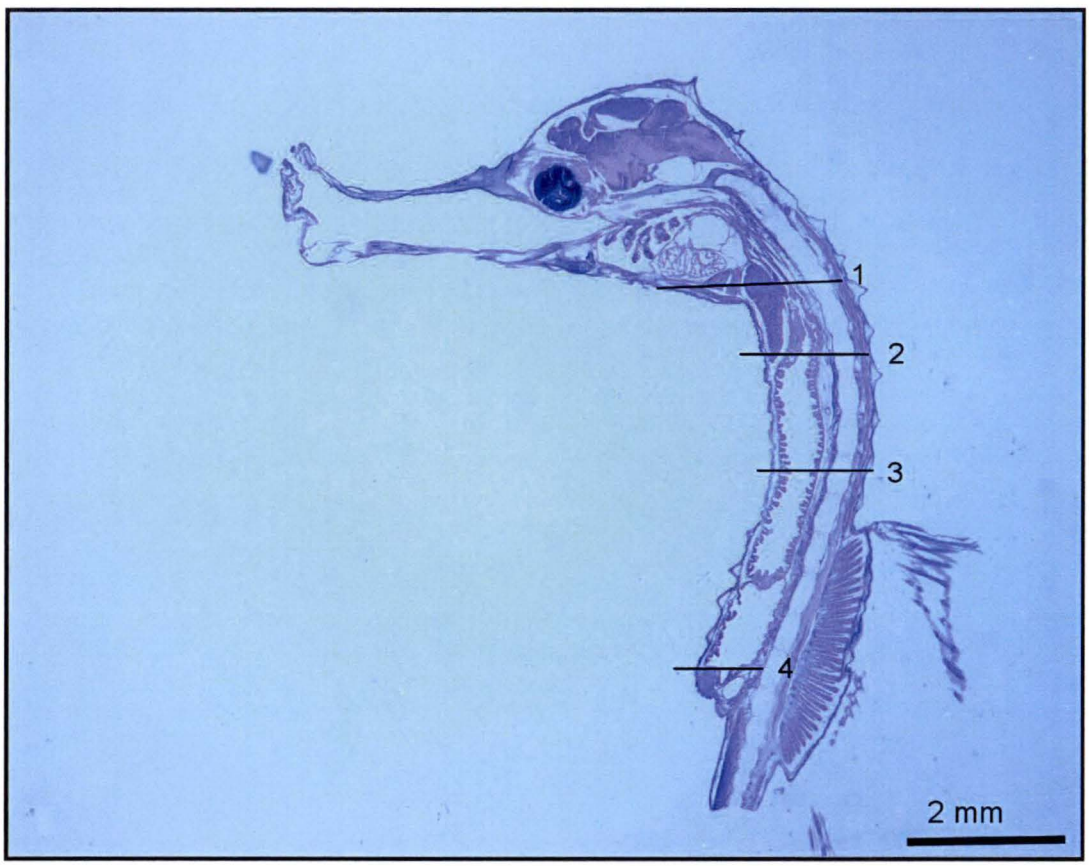
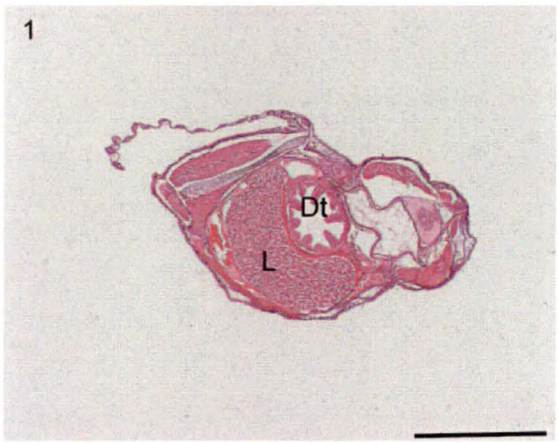
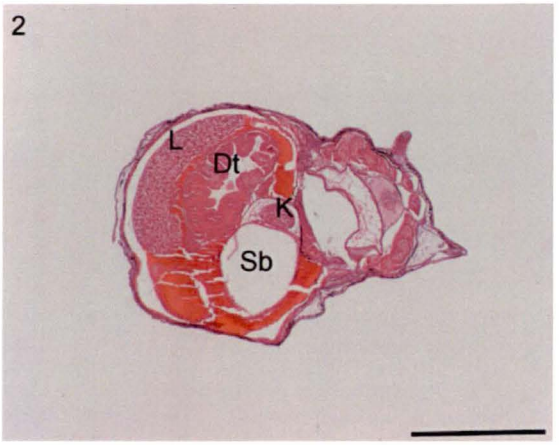
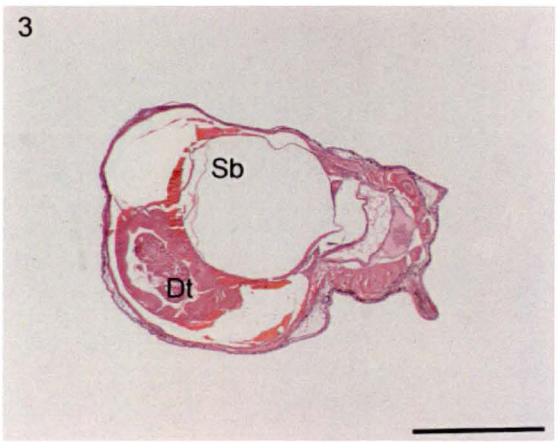


Figure 4.16. Approximate location of the transverse sections through a one day old seahorse stained with Haemotoxylin and Eosin. (1) The liver and top region of the digestive tract (scale, 50 μm). (2) The liver, top of the swim bladder, digestive tract and kidney (scale, 50 μm). (3) The swim bladder enlarging and the digestive tract (scale, 50 μm). (4) The lower region of the digestive tract and anus (scale, 50 μm). Legend: An, anus; Dt, digestive tract; K, kidney; L, liver; Sb, swim bladder.


1
Dt
L


2
L
Dt
K
Sb


3
Sb
Dt


4
An

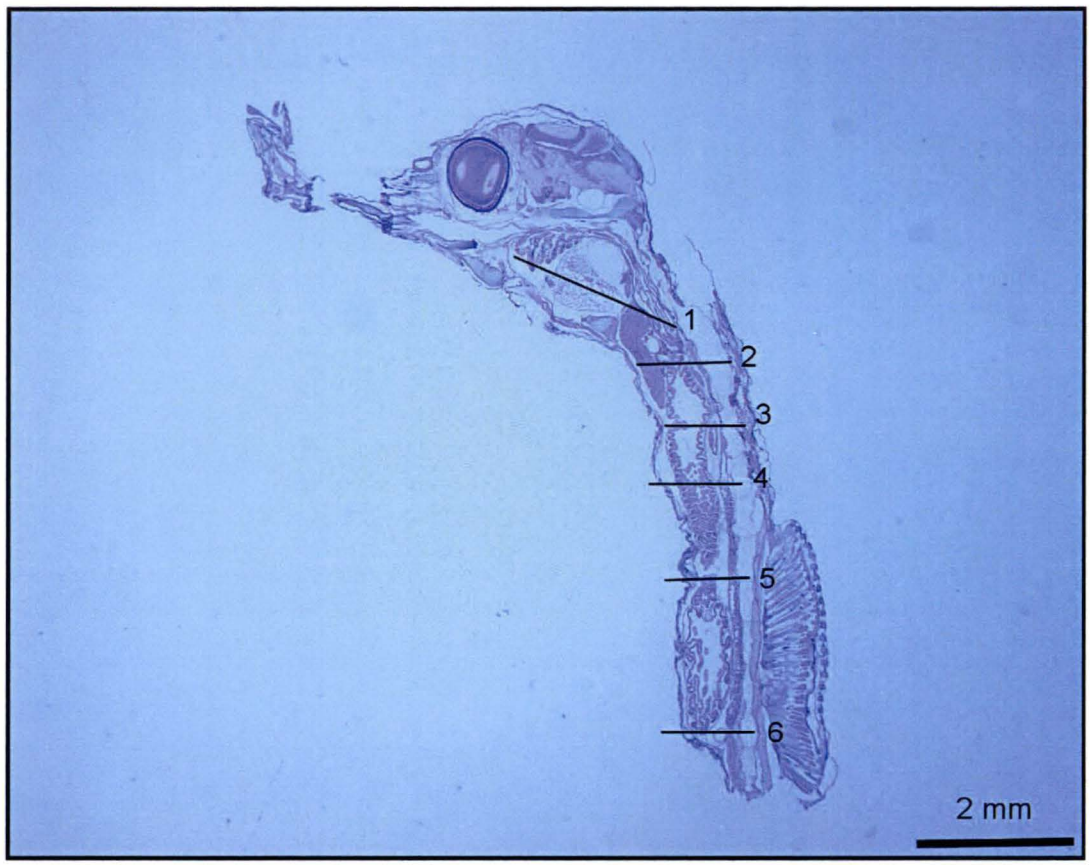
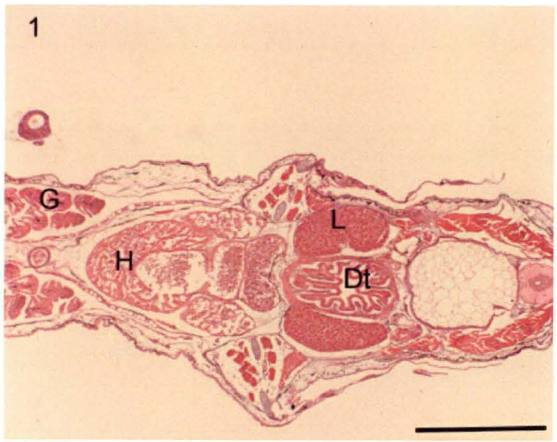
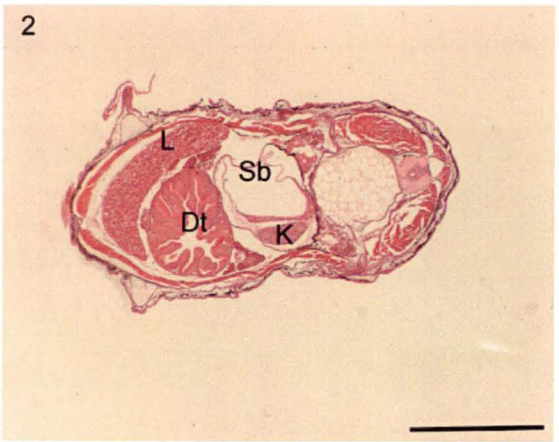
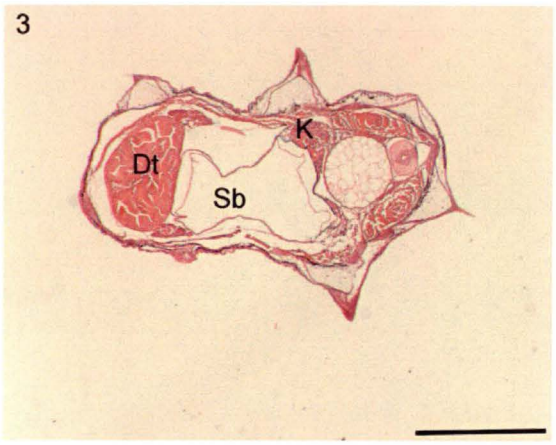
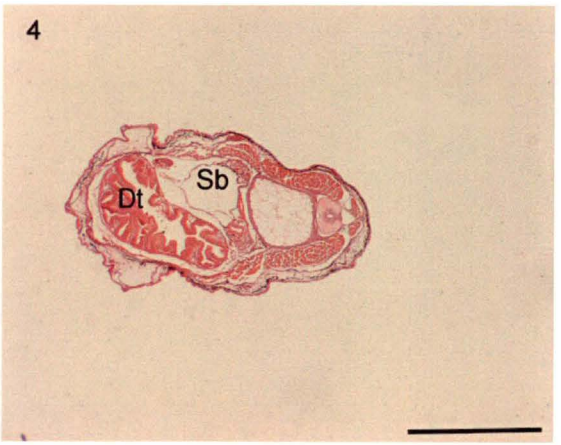


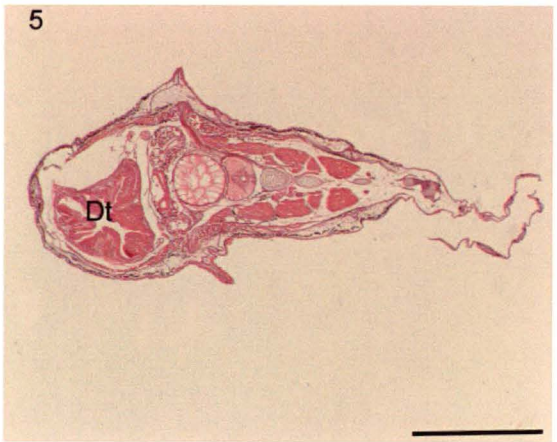
Figure 4.17. Approximate location of the transverse sections through a five day old seahorse stained with Haemotoxylin and Eosin. (1) The heart, liver and top region of the digestive tract (scale, 50µm). (2) The liver, swim bladder, kidney and top region of the digestive tract (scale, 50µm). (3) The swim bladder enlarging and the digestive tract (scale, 50µm). (4) The end of the swim bladder and the middle region of the digestive tract (scale, 50µm). (5) The lower digestive tract (scale, 50µm). (6) The lower digestive tract and anus of a 5 day old seahorse (scale, 50µm). Legend: An, anus; Dt, digestive tract; H, heart; K, kidney; L, liver; Sb, swim bladder.

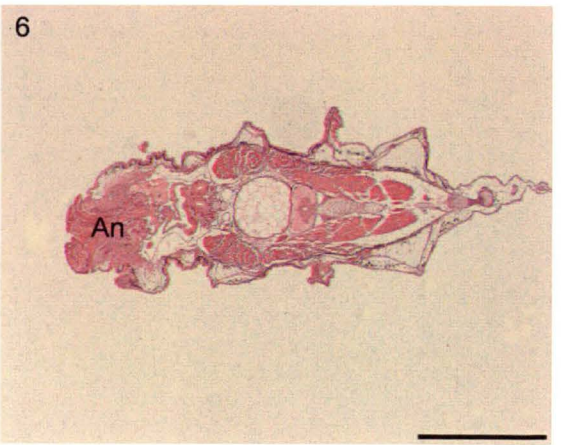

1
G
H
L
Dt


2
L
Sb
Dt
K


3
K
Dt
Sb


4
Sb
Dt


5
Dt


6
An

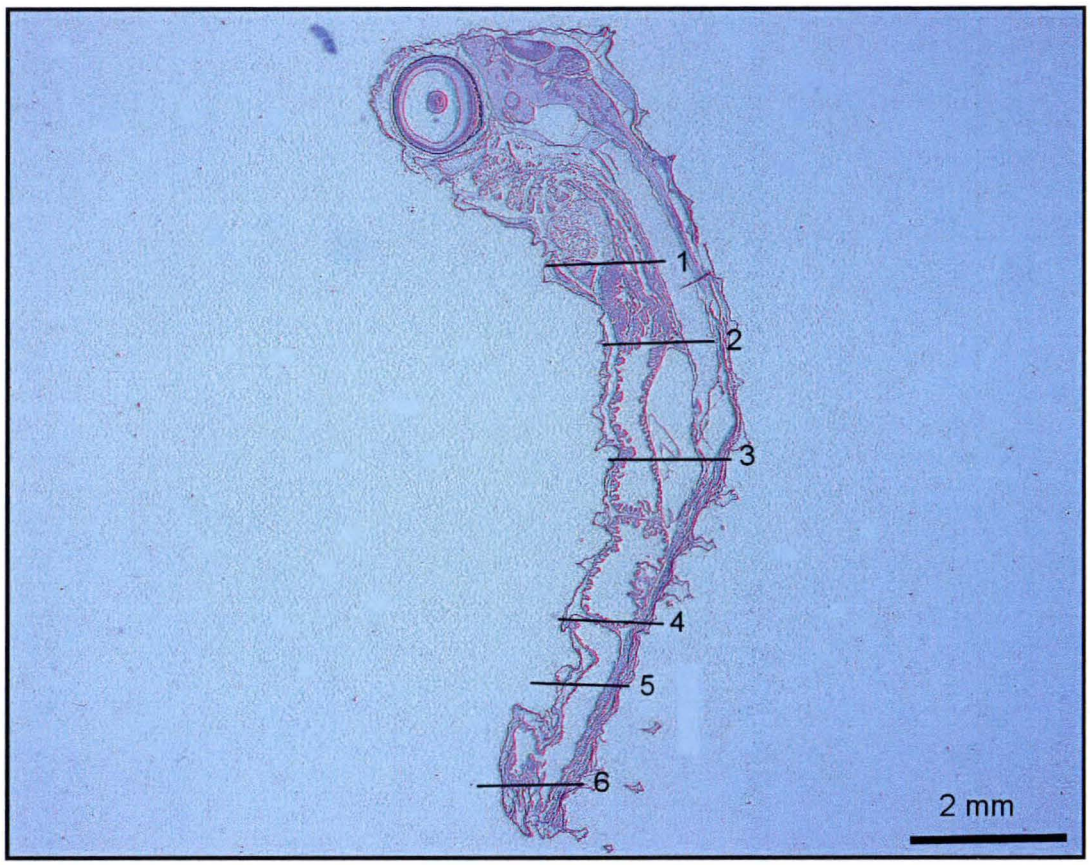
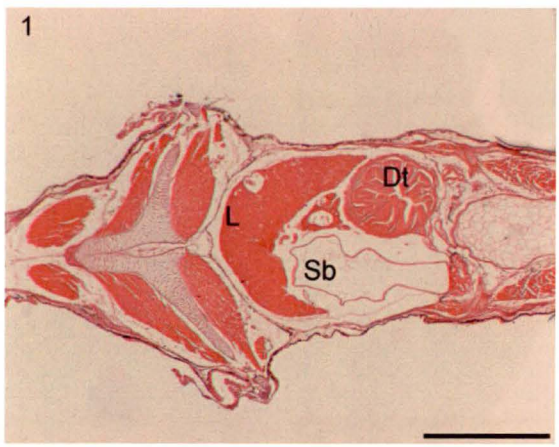
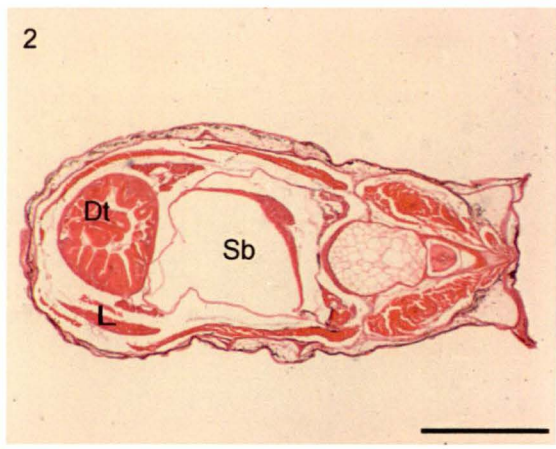
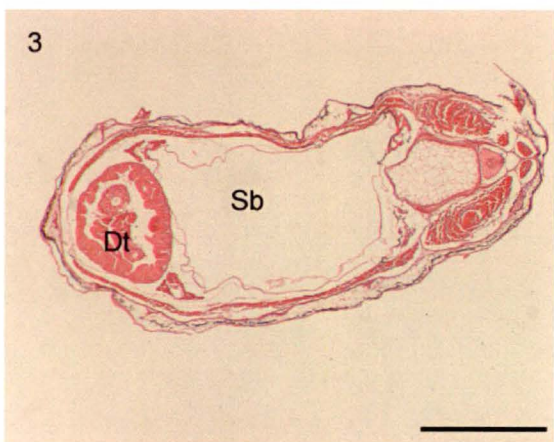
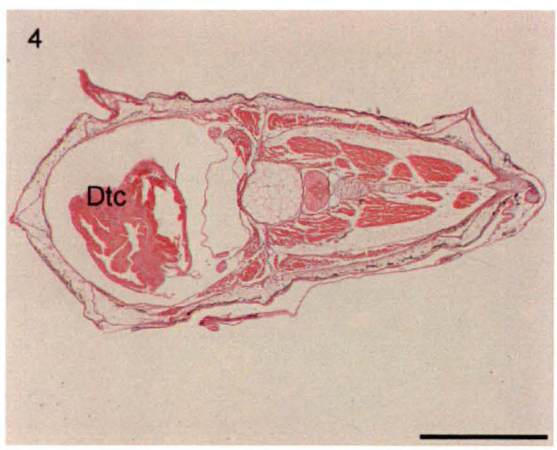


Figure 4.18. Approximate location of the transverse sections through a seven day old seahorse stained with Haemotoxylin and Eosin. (1) The liver, swim bladder and top region of the digestive tract (scale, 50μm). (2) The end of the liver, the swim bladder enlarging and the digestive tract (scale, 50μm). (3) The middle of the swim bladder and the digestive tract (scale, 50μm). (4) The constriction in the digestive tract (scale, 50μm). (5) The lower region of the digestive tract (scale, 50μm). (6) The lower region of the digestive tract and the anus (scale, 50μm). Legend: An, anus; Dt, digestive tract; Dtc, digestive tract constriction; K, kidney; L, liver; Sb, swim bladder.

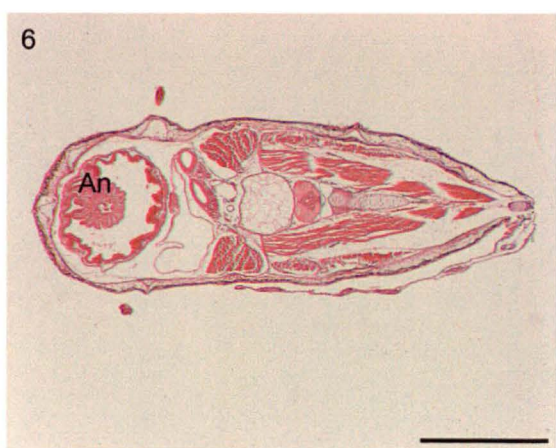

1
Dt
L
Sb


2
Dt
Sb
L


3
Sb
Dt


4
Dtc


5
Dt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An

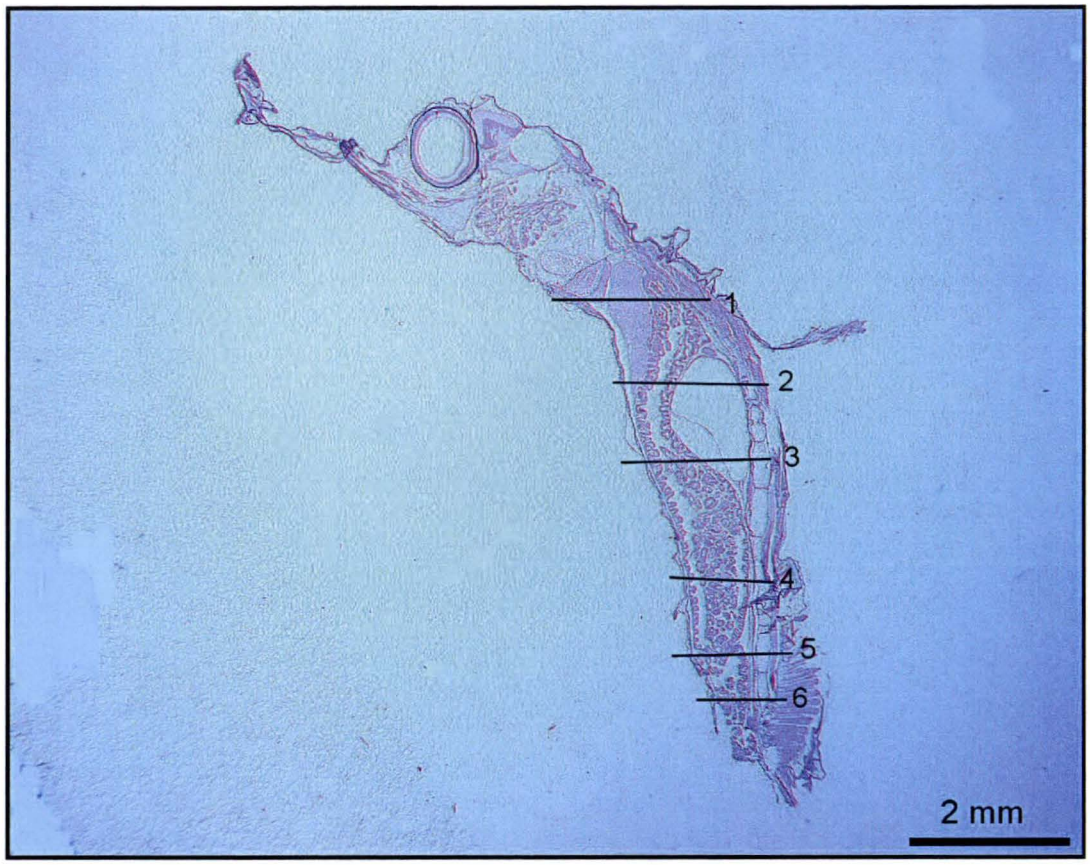
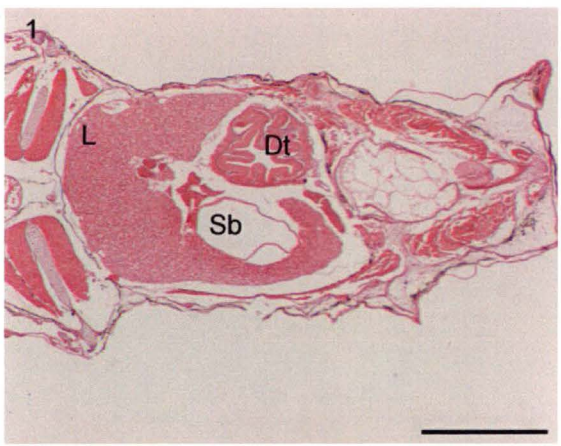


Figure 4.19. Approximate location of the transverse sections through a 14 day old seahorse stained with Haemotoxylin and Eosin. (1) The liver, swim bladder and top region of the digestive tract (scale, 50μm). (2) The middle of the swim bladder and the digestive tract (scale, 50μm). (3) The swim bladder and the digestive tract (scale, 50μm). (4) The kidney and middle region of the digestive tract (scale, 50μm). (5) The constriction in the digestive tract which leads to the lower region of the gut (scale, 50μm). (6) The lower region of the digestive tract (scale, 50μm). Legend: An, anus; Dt, digestive tract; Dtc, digestive tract constriction; K, kidney; L, liver; Sb, swim bladder.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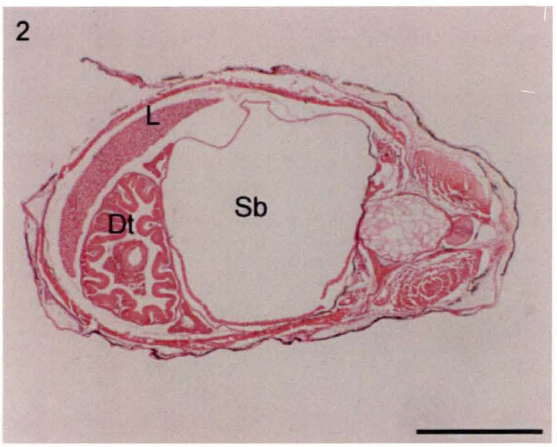
Dt
Sb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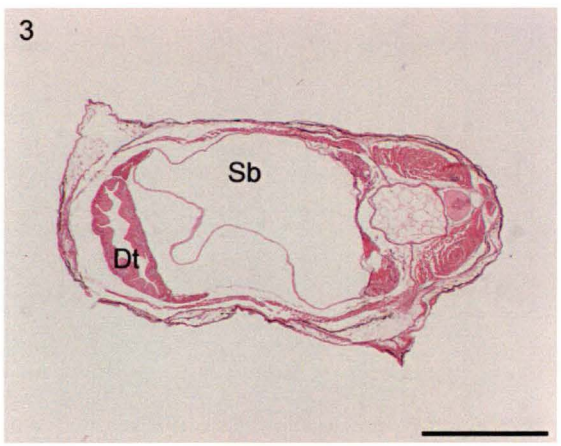
L
Sb
Dt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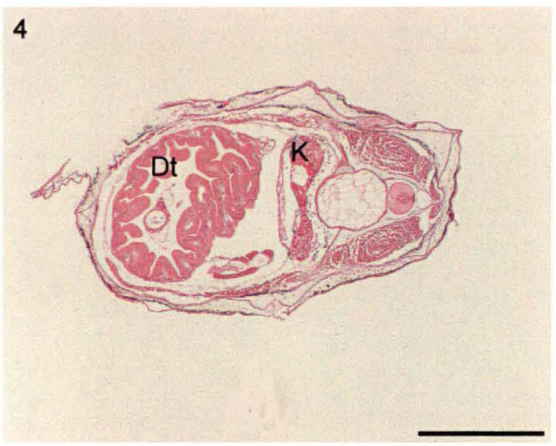
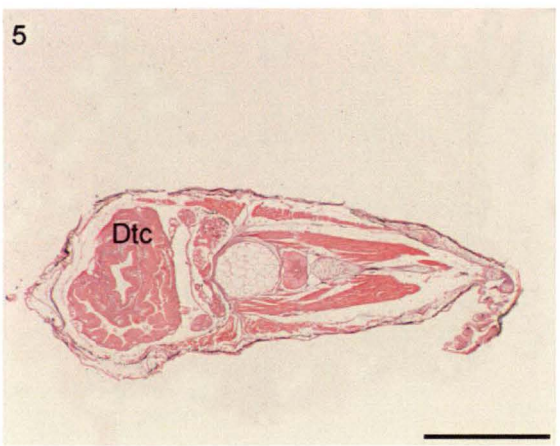

4
Dt
K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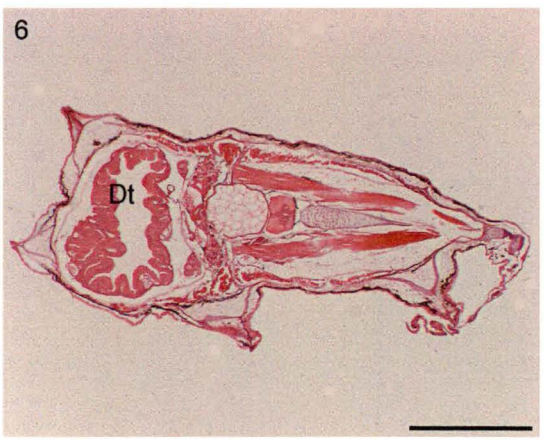
Dtc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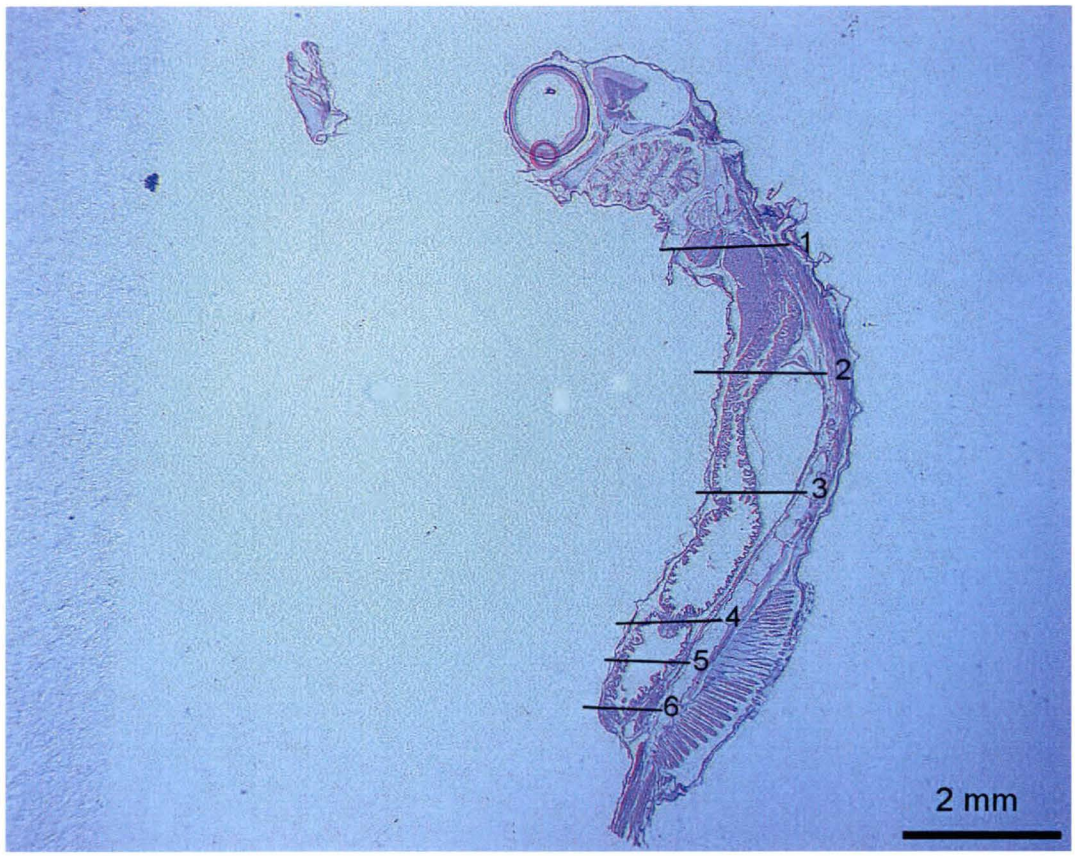
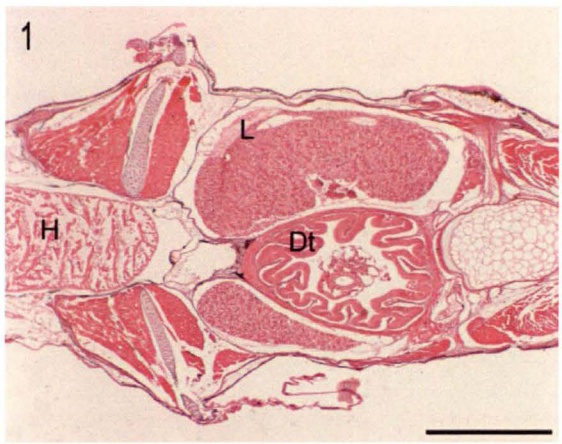


Figure 4.20. Approximate location of the transverse sections through a 21 day old seahorse stained with Haemotoxylin and Eosin. (1) The heart, liver and top region of the digestive tract (scale, 50µm). (2) The liver, swim bladder, kidney and top region of the digestive tract (scale, 50µm). (3) The swim bladder and the digestive tract (scale, 50µm). (4) The end of the swim bladder and the constriction in the digestive tract (scale, 50µm). (5) The lower region of the digestive tract (scale, 50µm). (6) The lower region of the digestive tract and the anus (scale, 50µm). Legend: An, anus; Dt, digestive tract; Dtc, digestive tract constriction; H, heart; K, kidney; L, liver; Sb, swim bladder.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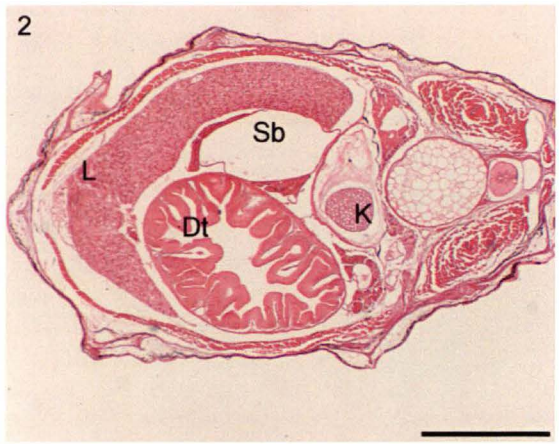
L
H
Dt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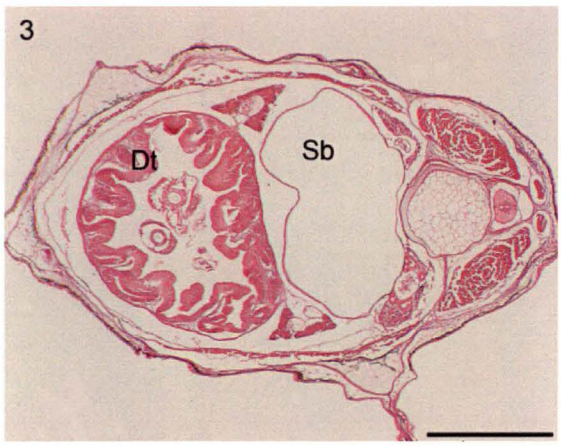
L
Dt
K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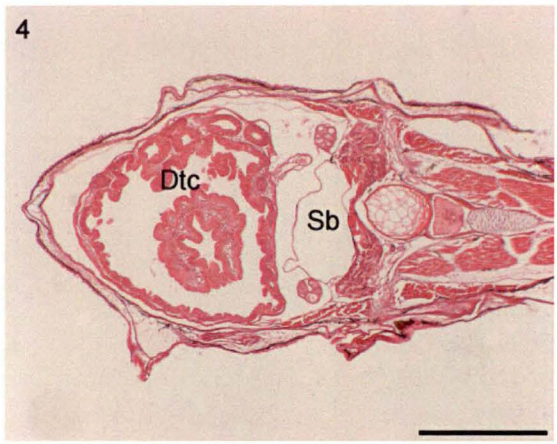
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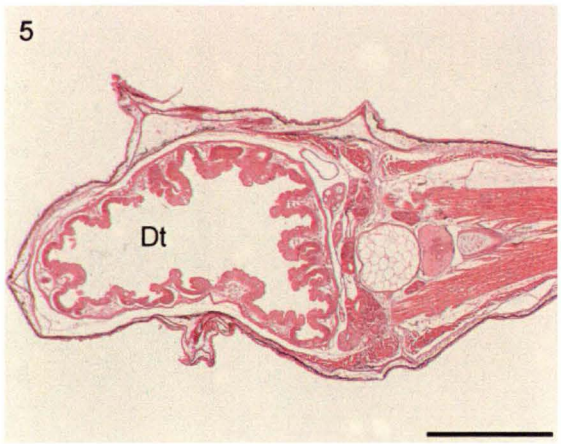
Dtc
Sb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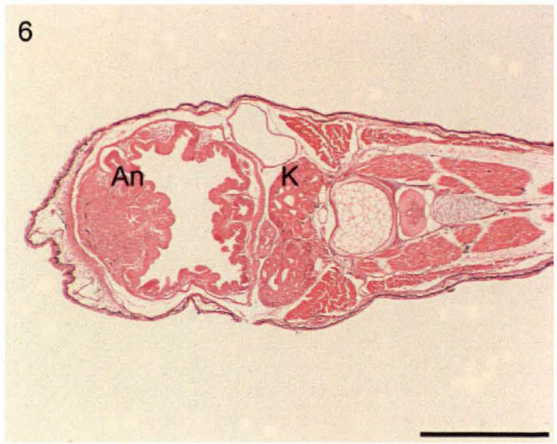
Dt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An
K

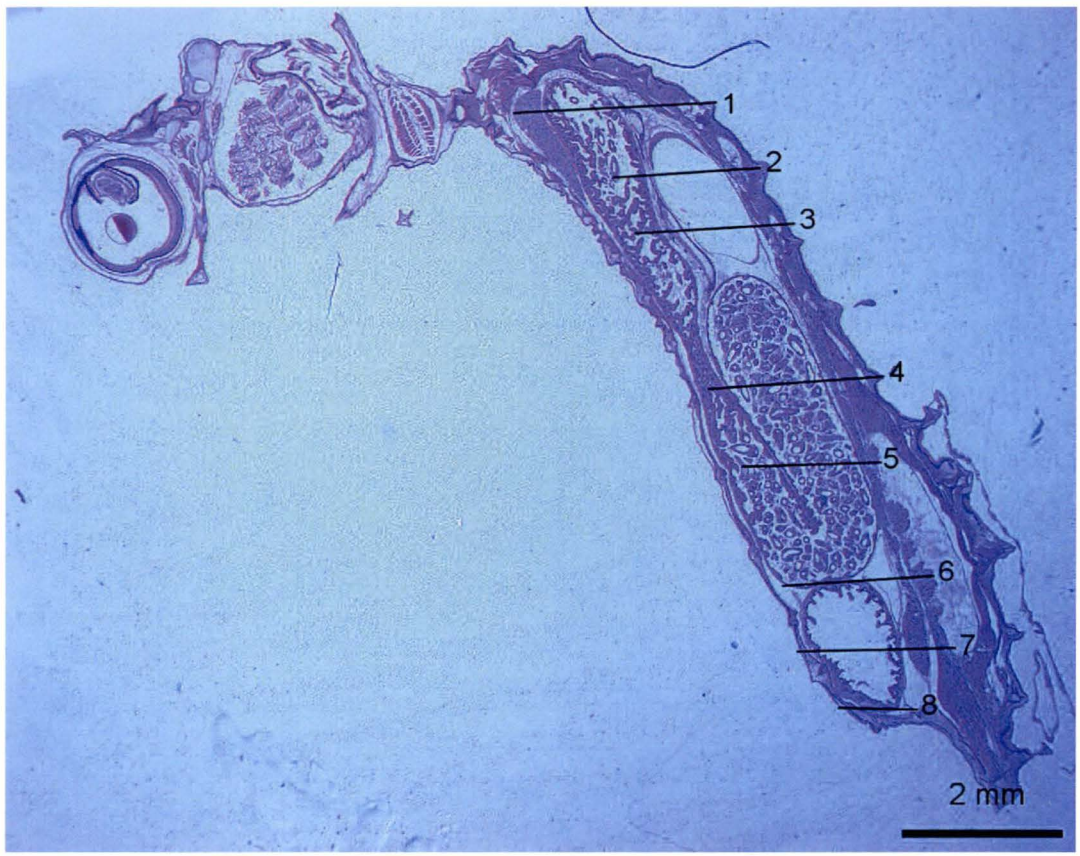
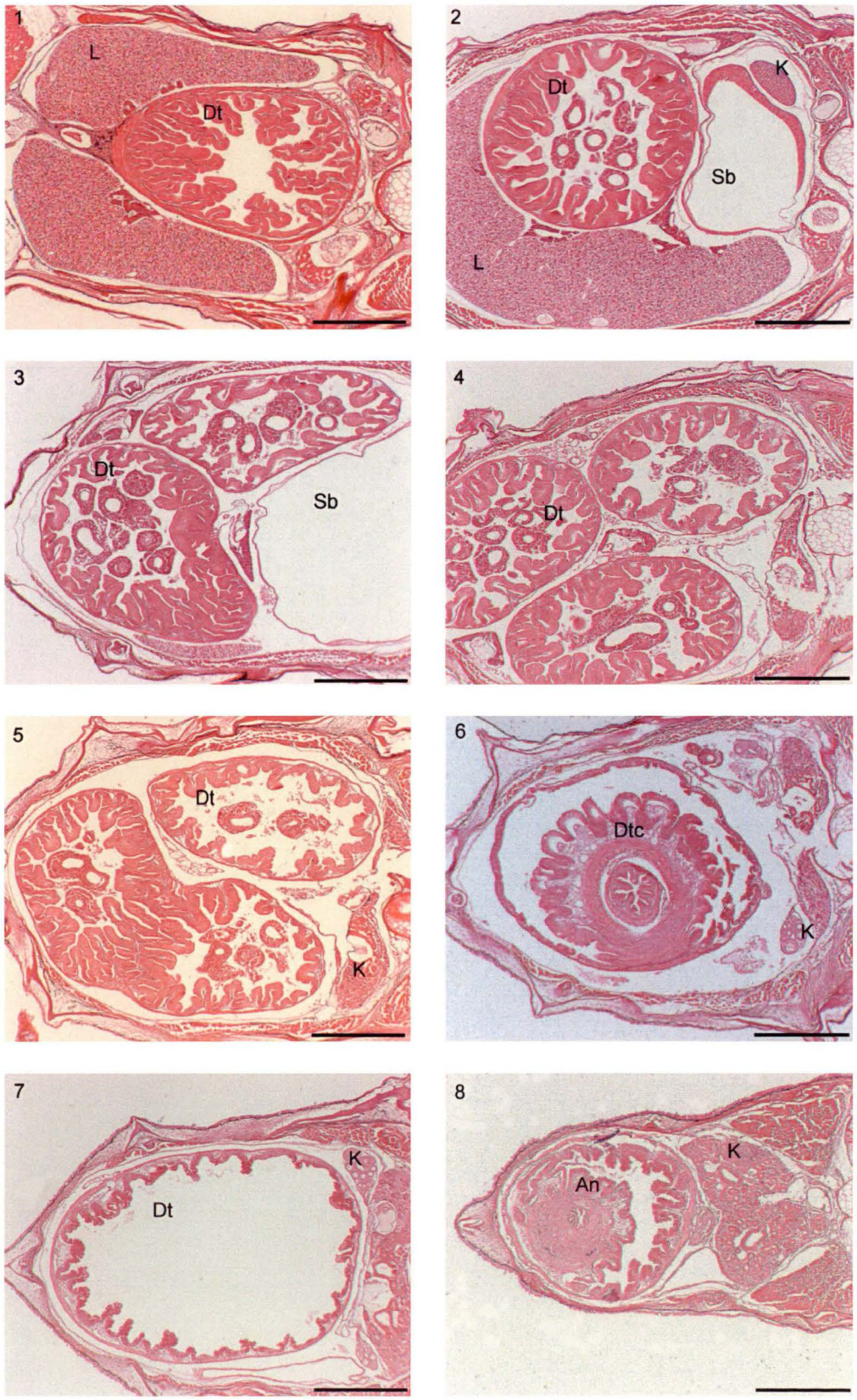


Figure 4.21. Approximate location of the transverse sections through a 35 day old seahorse stained with Haemotoxylin and Eosin. (1) The liver and top region of the digestive tract (scale, 50µm). (2) The liver, swim bladder and the digestive tract (scale, 50µm). (3) The swim bladder and loop in the top region of the digestive tract (scale, 50µm). (4) The loops in middle region of the digestive tract (scale, 50µm). (5) The loops in the middle region of the digestive tract (scale, 50µm). (6) The constriction in the digestive tract (scale, 50µm). (7) The lower region of the digestive tract (scale, 50µm). (8) The anus of the seahorse. Legend: An, anus; Dt, digestive tract; Dtc, digestive tract constriction; K, kidney; L, liver; Sb, swim bladder.


1
L
Dt
2
Dt
K
Sb
L
3
Dt
Sb
4
Dt
5
Dt
K
6
Dtc
K
7
K
Dt
8
K
An

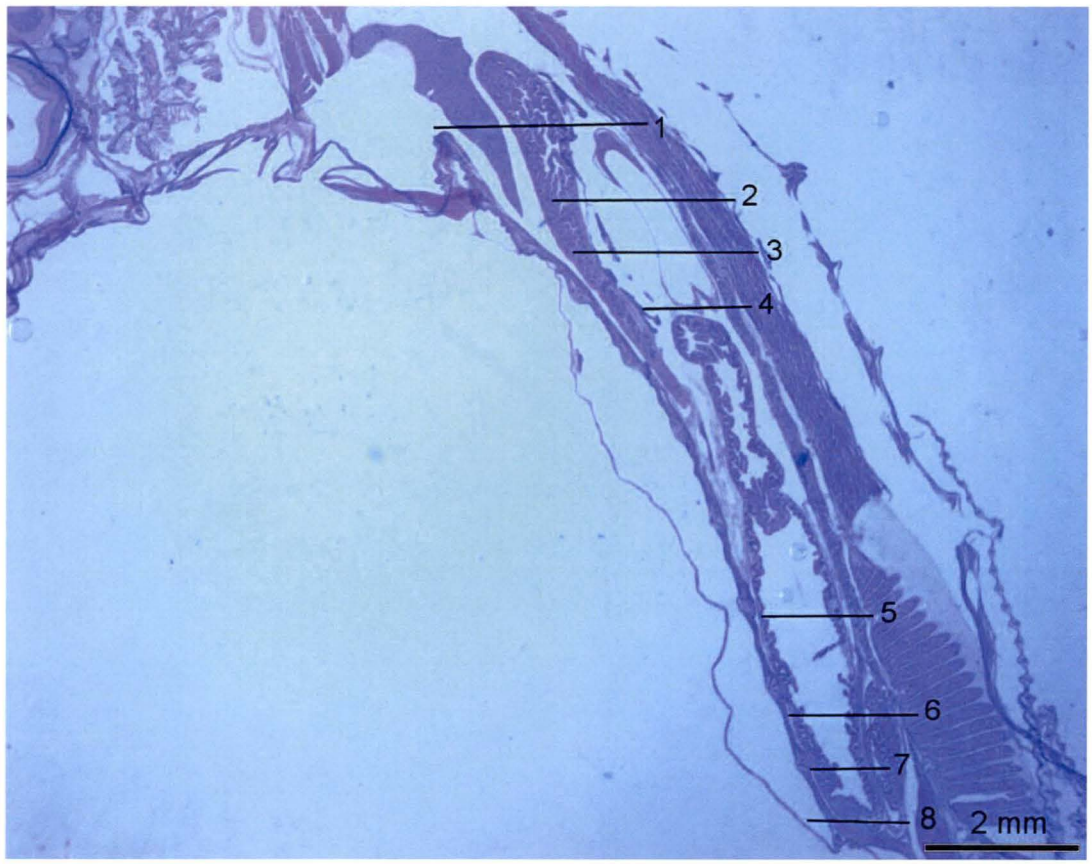
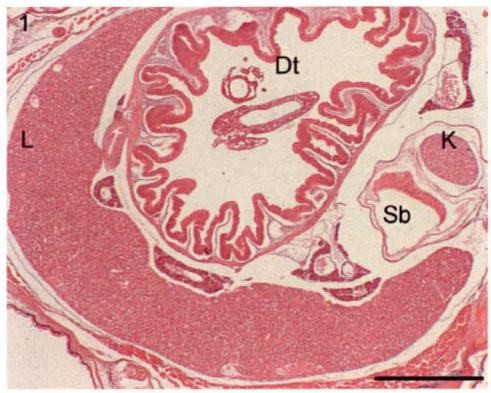
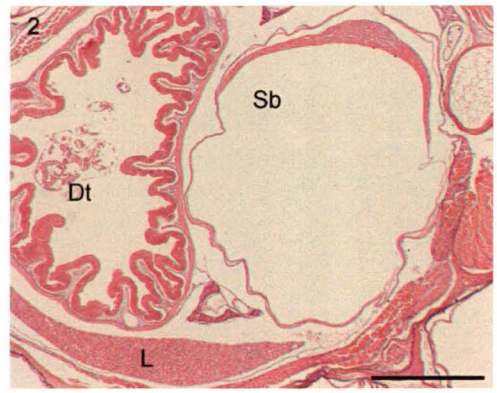


Figure 4.22. Approximate location of the transverse sections through a 49 day old seahorse stained with Haemotoxylin and Eosin. (1) The liver, swim bladder, kidney and top region of the digestive tract (scale, 50μm). (2) The swim bladder and the top region of the digestive tract (scale, 50μm). (3) The swim bladder and the loops in the digestive tract (scale, 50μm). (4) The loops in the digestive tract (scale, 50μm). (5) The digestive tract (scale, 50μm). (6) The middle region and the lower region of the digestive tract (scale, 50μm). (7) The lower region of the digestive tract (scale, 50μm). (8) The lower region of the digestive tract leading to the anus (scale, 50μm). Legend: An, anus; Dt, digestive tract; K, kidney; L, liver; Sb, swim bladder


1
Dt
L
K
Sb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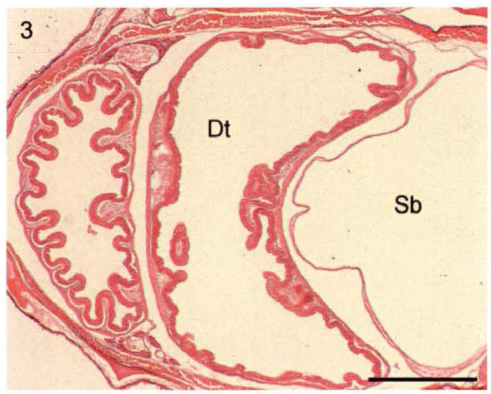
Sb
Dt
L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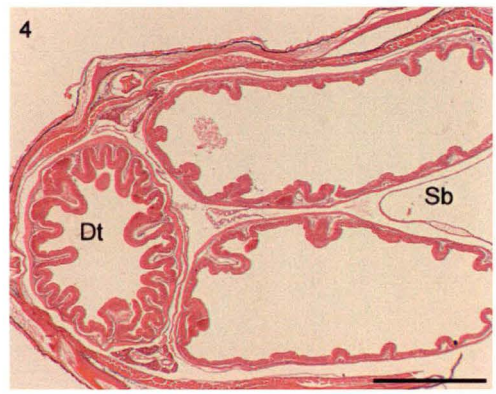
Dt
Sb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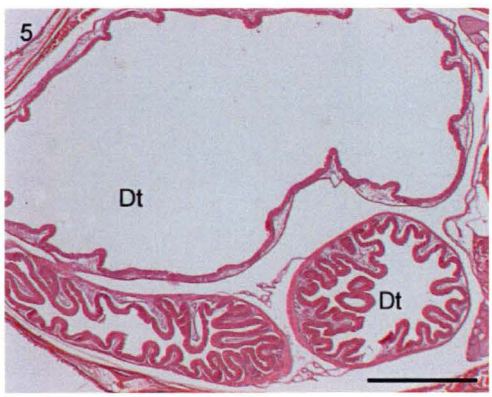
Sb
Dt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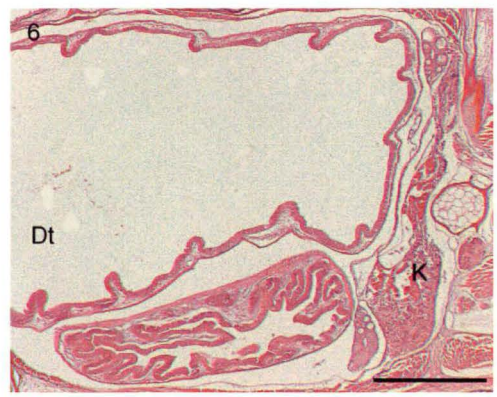
Dt
Dt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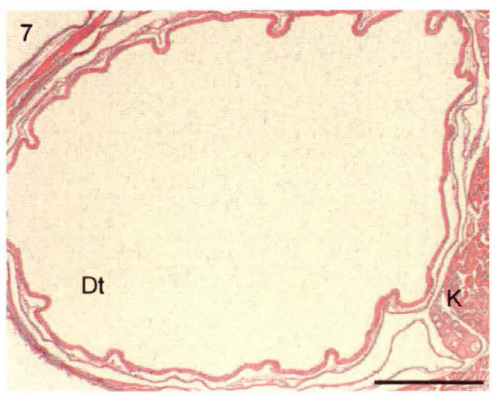
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Dt
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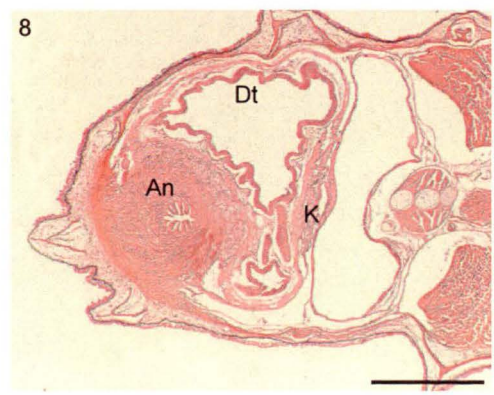

8
Dt
An
K

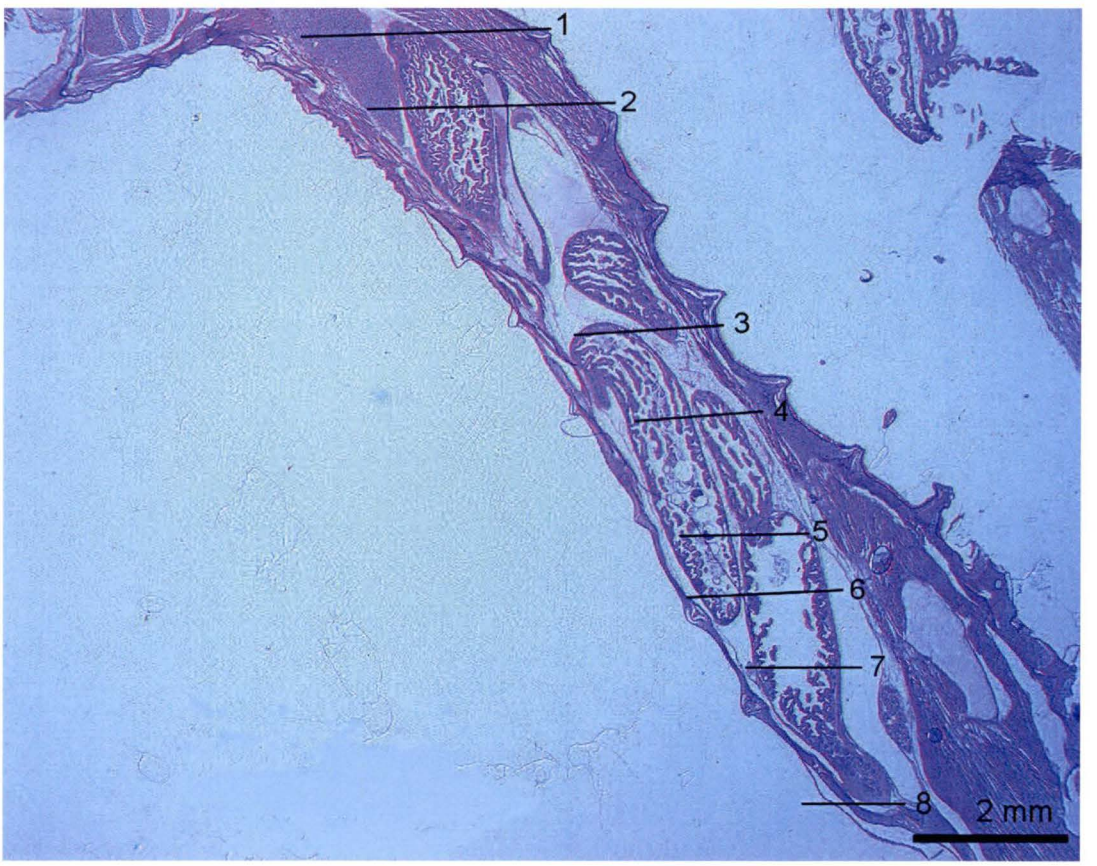
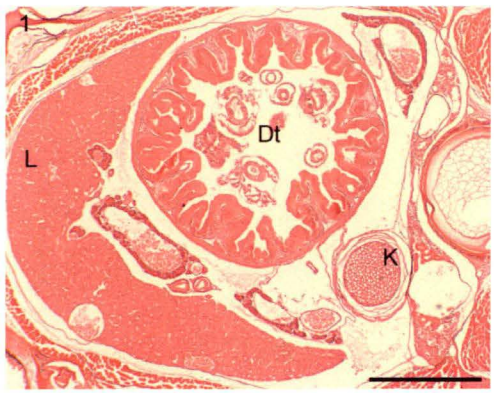


Figure 4.23. Approximate location of the transverse sections through a 56 day old seahorse stained with Haemotoxylin and Eosin. (1) The liver, kidney and top region of the digestive tract (scale, 50µm). (2) The swim bladder, kidney and the top region of the digestive tract (scale, 50µm). (3) The loops in the middle region of the digestive tract (scale, 50µm). (4) The loops in the digestive tract (scale, 50µm). (5) The loop in the digestive tract and the constriction in the gut leading to the lower gut (scale, 50µm). (6) The middle region and the lower region of the digestive tract (scale, 50µm). (7) The lower region of the digestive tract and the kidney (scale, 50µm). (8) The anus of the seahorse (scale, 50µm). Legend: An, anus; Dt, digestive tract; Dtc, digestive tract constriction; K, kidney; L, liver; Sb, swim bladder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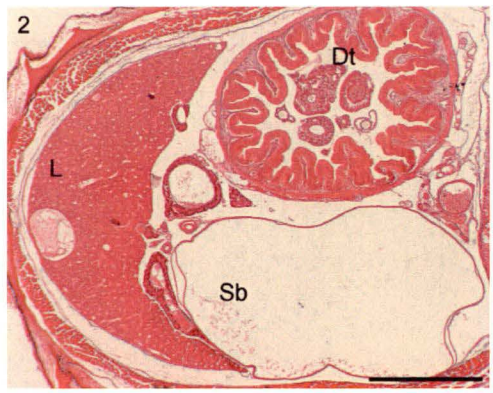
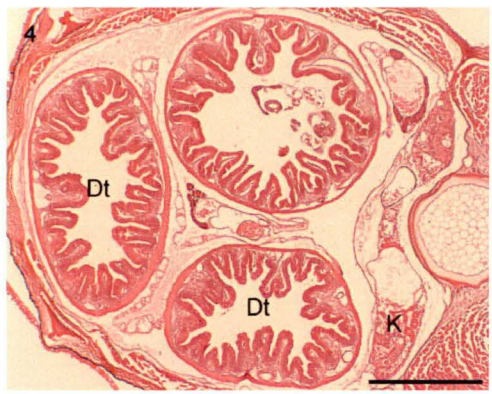
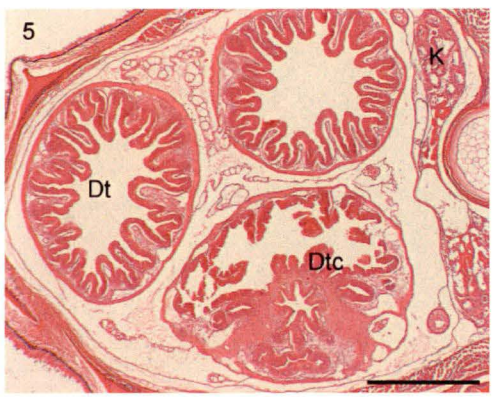
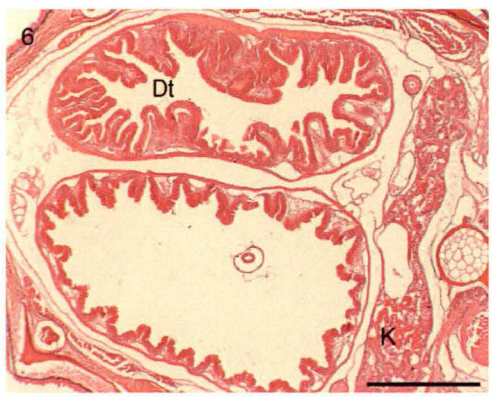
L
K


2
Dt
L
Sb


3
Dt
K


4
Dt
Dt
K


5
K
Dt
Dtc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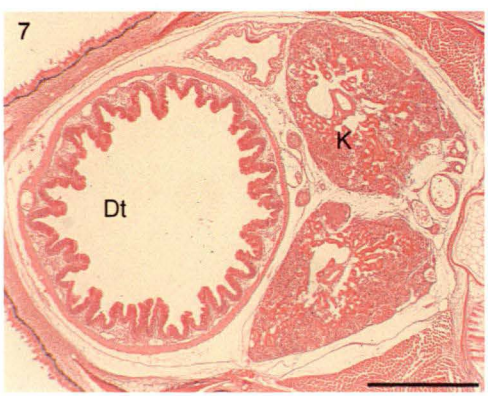
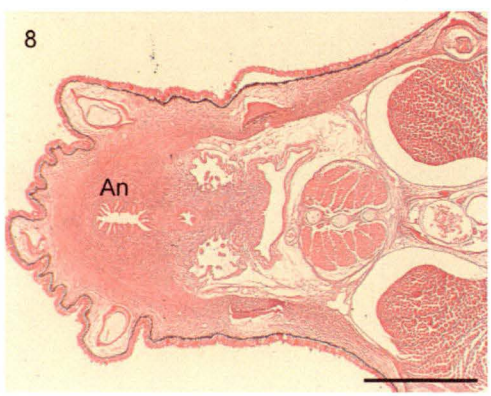
Dt
K


7
K
Dt


8
An

4.3.2. CELL TYPES OF THE DIGESTIVE TRACT AND EXTRAMURAL ORGANS

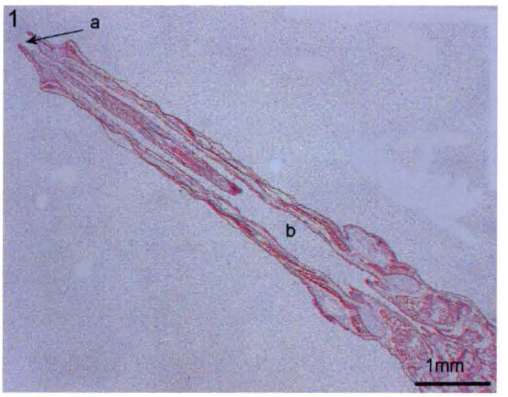
Digestive tract

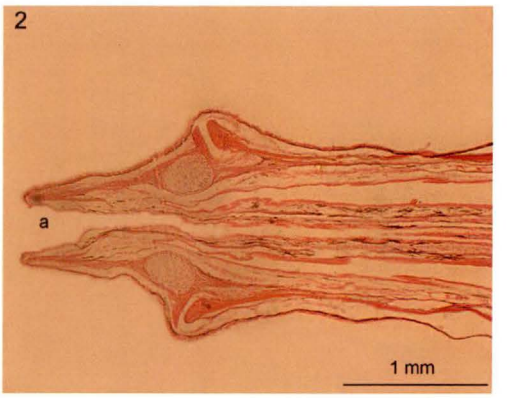
A seahorse has a long snout, which allows for suction feeding and food is passed into the oesophagus past the gills. The oesophagus of a seahorse is composed of 4 layers, the mucosa, submucosa, muscularis and outer serosa. The mucosa layer rests on a basement membrane, is folded longitudinally and comprises stratified squamous epithelium cells and goblet cells. The submucosa consists of loose connective tissue and the muscularis layer is comprised of circular muscle (Figure 4.24). A short section of the oesophagus, proximal to the valve, which leads directly into the intestine, shows a distinct difference in cell type with the epithelium abruptly changing to a columnar type. Also, within the epithelium there appear to be the equivalent of gastric cells (Figure 4.25) as found in the stomach of most other fish species.

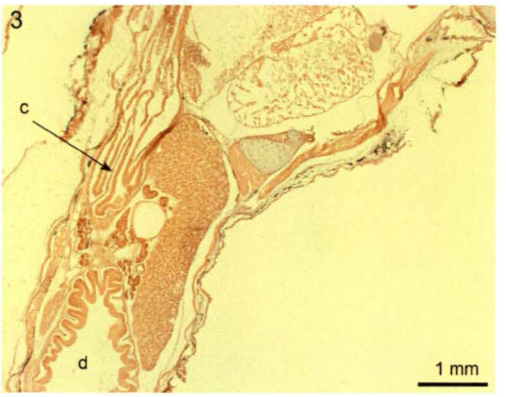
The intestine of a seahorse is composed of a mucosa layer which is comprised of columnar epithelium and goblet cells, a submucosa layer which is separated from the mucosa layer by lamina propria and a muscularis layer where there is an inner circular muscle layer that is thicker than the outer longitudinal muscle layer. It is also found that there are regions of mucous secreting cells located within the submucosa at the base of the intestinal folds (Figure 4.26).

The intestinal valve of a seahorse has a mucosa layer which closely resembles the mucosa of the digestive tract and a thick layer of circular muscle, which is discontinuous. It was also noted that there is no longitudinal muscle around the valve (Figure 4.27).

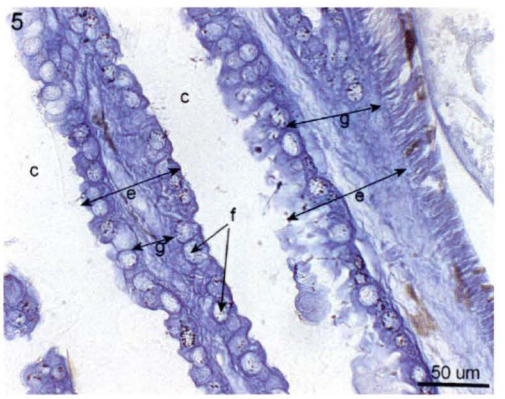
Figure 4.24. Sections showing the buccal cavity and oesophagus of seahorses. (1) Transverse section of the mouth and buccal cavity of a 35 day old seahorse. (2) Transverse section of the mouth of a 35 day old seahorse. (3) Longitudinal section of a 5 day old seahorse showing the oesophagus and the top region of the digestive tract. (4) Longitudinal section of a 14 day old seahorse showing the oesophagus. (5) Transverse section of a 56 day old seahorse showing the digestive tract. (6) Transverse section of a 56 days old seahorse showing the intestinal mucosa of the digestive tract. Legend: (a) mouth; (b) buccal cavity; (c) oesophagus; (d) intestine; (e) mucosa; (f) oesophageal goblet cell; (g) stratified squamous epithelium; (h) basement membrane.


1
a
b
1mm


2
a
1 mm

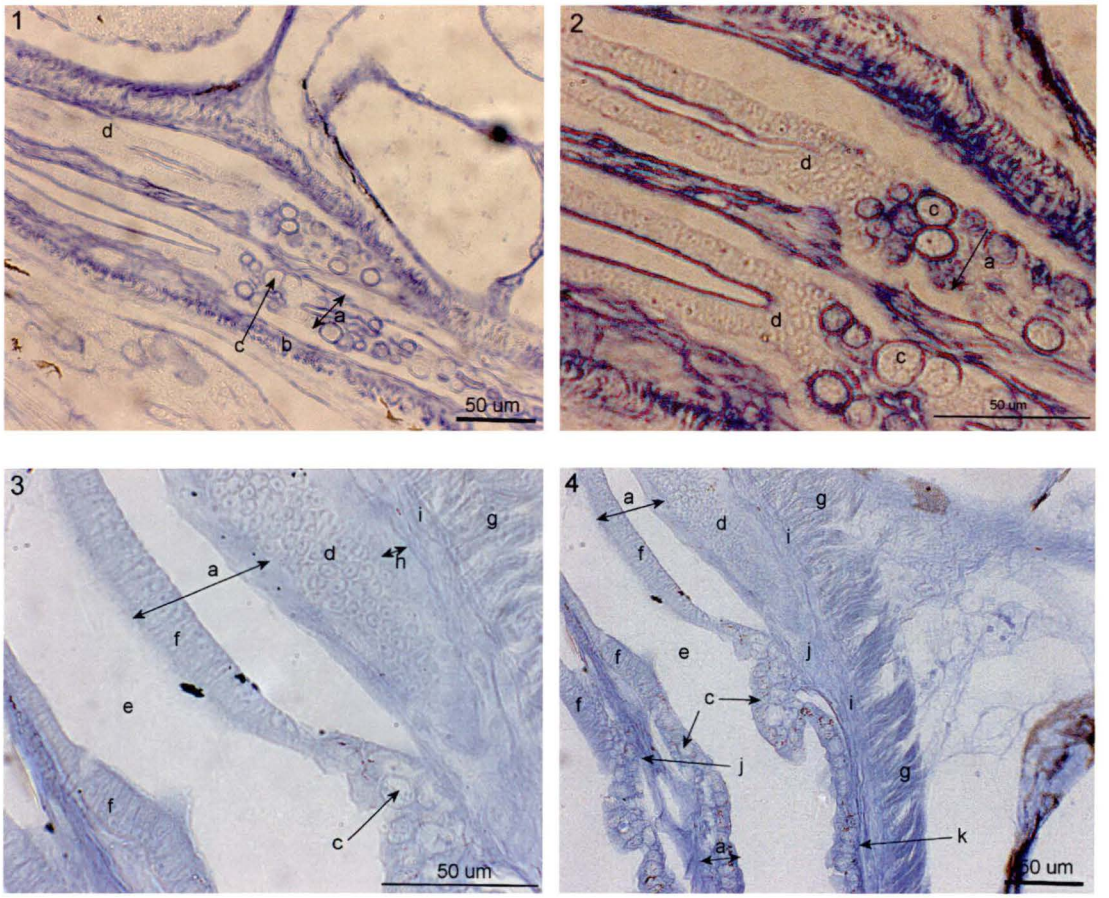

3
c
d
1 mm


4
h
e
g
f
c
20 um


5
c
c
g
e
e
f
g
50 um


6
c
d
2 mm

Figure 4.25. Longitudinal sections stained with Mecuro bromophenol blue showing the gastric cells within the caudal region of the oesophagus. (1) Gastric cells in newborn seahorses. (2) Gastric cells in newborn seahorses. (3) Gastric cells in 56 day old seahorses. (4) Gastric cells in 56 day old seahorses. Legend: (a) mucosa; (b) stratified squamous epithelium; (c) oesophageal goblet cell (type A); (d) gastric cells; (e) oesophagus lumen; (f) columnar epithelial cells; (g) circular muscle; (h) submucosa; (i) longitudinal muscle; (j) areolar connective tissue; (k) stratum compactum.


1
d
a
c
b
50 um
2
d
c
a
d
c
50 um
3
i
g
d
h
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f
e
f
c
50 um
4
a
g
d
i
f
f
e
j
c
i
j
g
a
k
50 um

Figure 4.26. Sections showing the intestinal wall of seahorses. (2) Longitudinal section of a 35 day old seahorse stained with Mecuro bromophenol blue showing the laminia propria and mucosal epithelium. (3) Transverse section of a 56 day old seahorse showing the intestinal mucosa of the digestive tract. Legend: (a) muscular layer; (b) longitudinal muscle; (c) circular muscle; (d) submucosa; (e) mucosal epithelium; (f) lamina propria; (g) columnar epithelium cells; (h) goblet cells; (i) mucous secreting cells.


1
e
d
c
b
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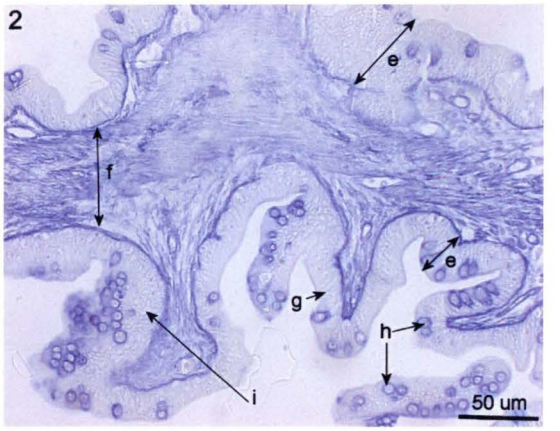
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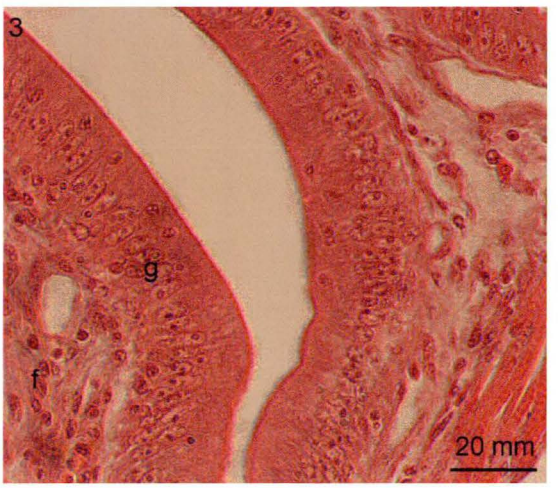
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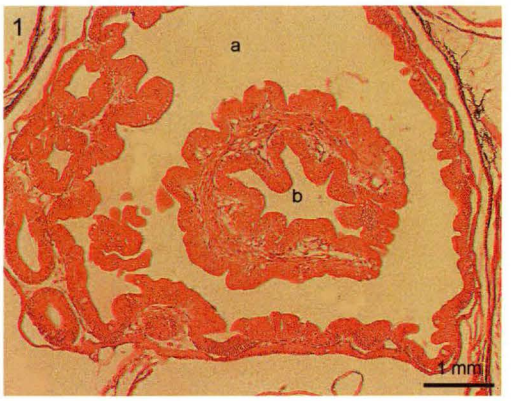
Figure 4.27. Transverse sections stained with Haemotoxylin and Eosin of the intestinal valve, lower gut and anus of the pot bellied seahorse. (1) The intestinal valve separating the intestine from the rectum in a 21 day old seahorse. (2) The intestinal valve of a 21 day old seahorse. (3) The rectum of a newborn seahorse. (4) The rectum of a 56 day old seahorse. (5) The rectal wall of a 21 day old seahorse. (6) The anal opening of a 49 day old seahorse. Legend: (a) intestine; (b) intestinal valve; (c) circular muscle layer; (d) mucosal epithelium; (e) columnar epithelium; (f) rectum; (g) submucosa; (h) goblet cell; (i) longitudinal muscle layer; (j) anal opening.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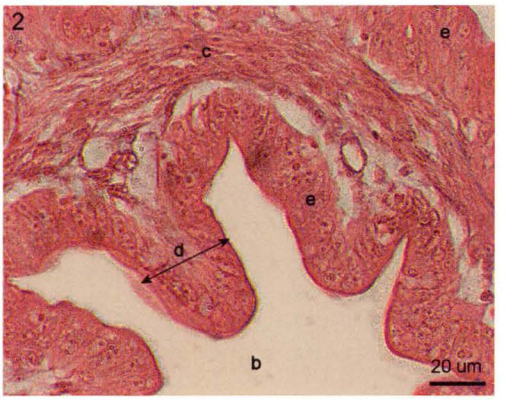
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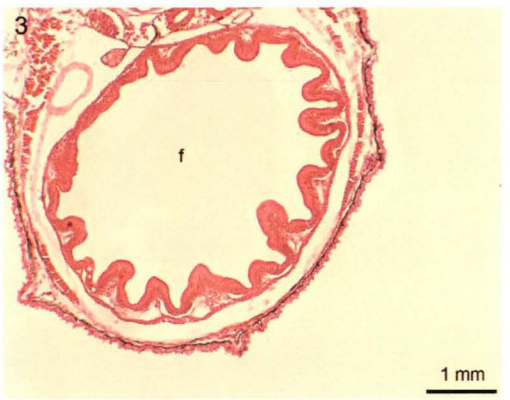
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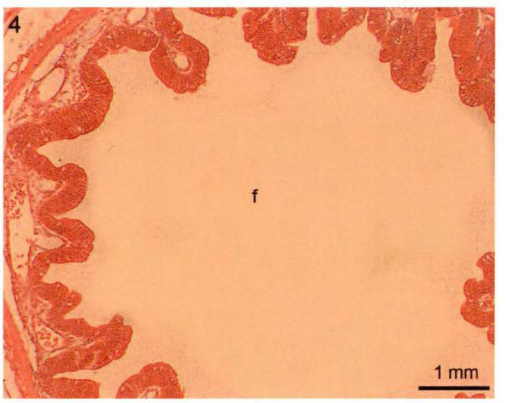
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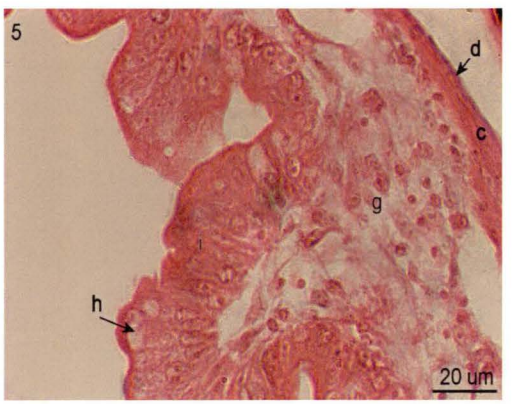
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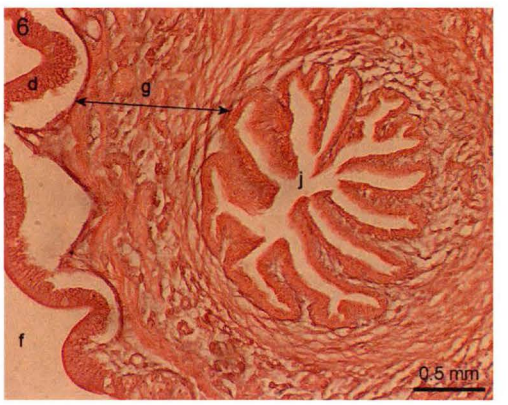
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The wall of the rectum is similar in composition to the wall proximal to the valve except that the mucosal folds which were quite long are now shorter and flatter, the muscularis layer has decreased and there is a greater occurrence of goblet cells. The digestive tract ends at the anus and at the anal opening the circular muscle layer is absent. The connective tissue layer expands and surrounds the anus and this joins to the body wall (Figure 4.27).

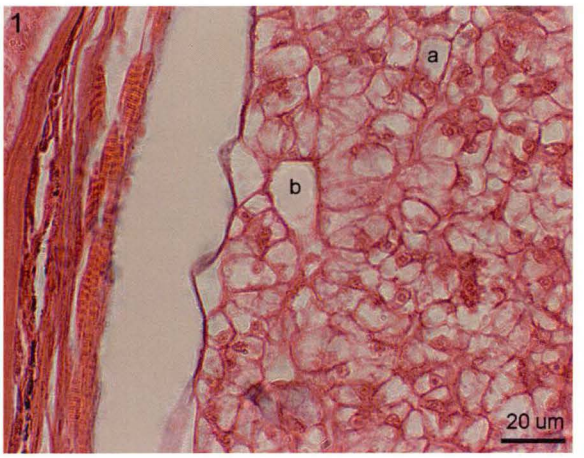
Extramural organs

The liver is composed of a meshwork of hepatocytes, which make up hepatic units. Other features of the liver are veins, accumulations of tissue macrophages and exocrine pancreatic tissue, found near the veins (Figure 4.28). Both the exocrine and endocrine pancreatic tissue of seahorses are composed of acini cells, which in groups make up the acinus of a pancreas and have interlobular pancreatic ducts. The bile from the gall bladder together with mucosubstances and enzymes from the pancreas are transferred to the intestine via the common bile duct (Figure 4.29)

4.3.3. Development of the digestive tract over time

On release from the pouch, the mouth and anus of a seahorse are open and the intestine is a simple, straight tube composed of a mucosa, submucosa, muscularis and outer serosa layer. The cells, which appear to be gastric cells, are present in the oesophagus, the clusters of mucus secreting cells are present in the epithelium of the intestine and the liver and pancreas are already formed. Over time there is very little change except that an intestinal valve separating the rectum from the rest of the intestine started to appear on day 5; the intestine began to loop around itself between day 21 and day 35 and the mucosa layer of the intestine was found to get thicker and more complex with age (Figure 4.30).

Figure 4.28. Transverse sections stained with Haemotoxylin and Eosin of the extramural organs (liver and pancreas) of a pot-bellied seahorse. (1) The central vein in the liver of a 35 day old seahorse. (2) An aggregation of tissue macrophages in the liver of a 56 day old seahorse. (3) Exocrine pancreatic tissue and liver of a 56 day old seahorse. (4) Exocrine pancreatic tissue and interlobular pancreatic duct of a 49 day old seahorse. Legend: (a) hepatocytes; (b) central vein; (c) tissue macrophages; (d) pancreatic tissue; (e) acinus of the pancreas; (f) interlobular pancreatic duct.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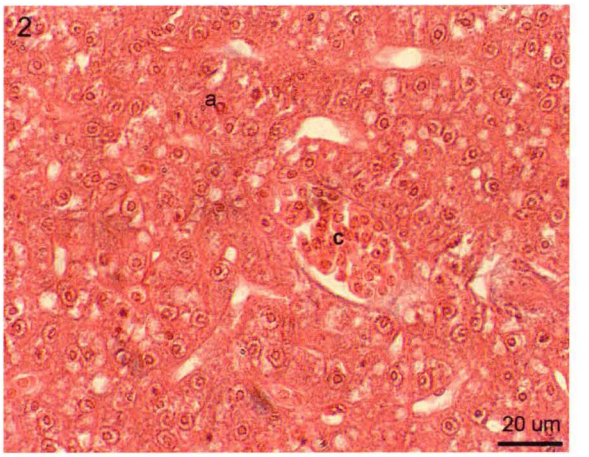
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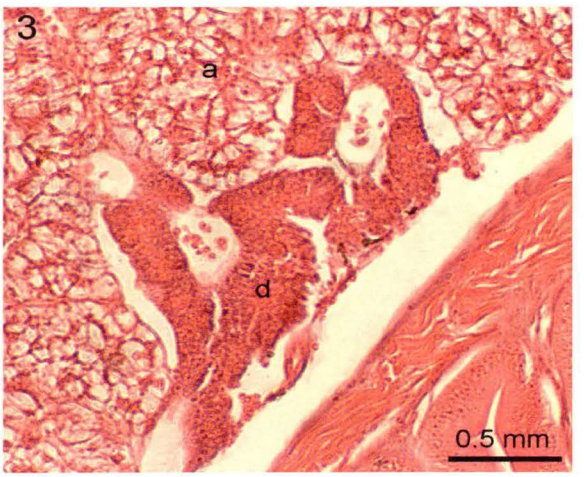
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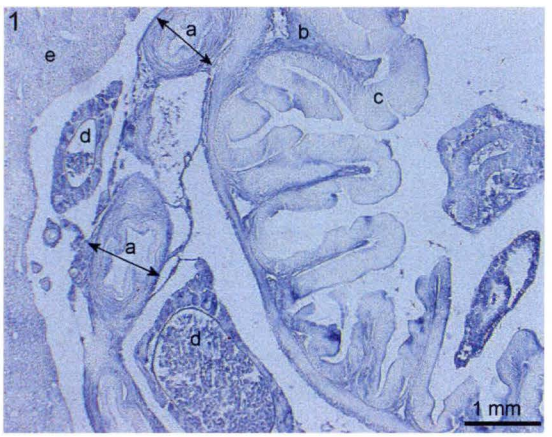
Figure 4.29. Transverse sections stained with Mecuro bromophenol blue (1) Ductus choledochus of a 56 day old seahorse. (2) Ductus choledochus of a 56 day old seahorse. (3) Ductus choledochus of a 21 day old seahorse. Legend: (a) ductus choledochus; (b) laminia propria; (c) mucosal epithelium; (d) exocrine pancreatic tissue; (e) liver; (f) muscularis; (g) columnar epithelium; (h) submucosa.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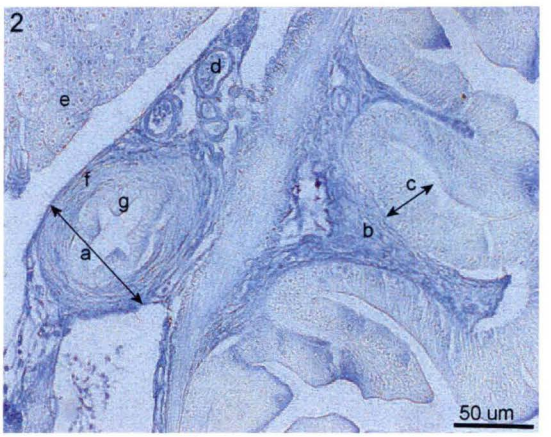
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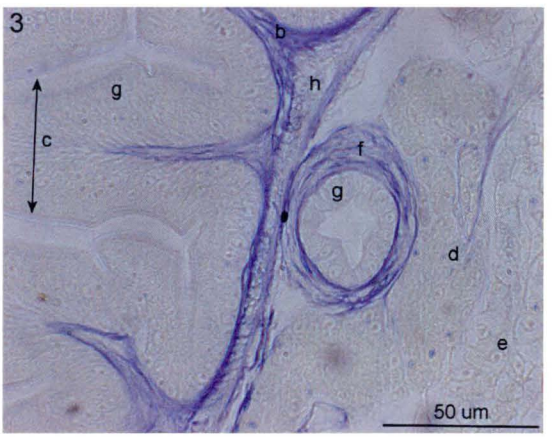
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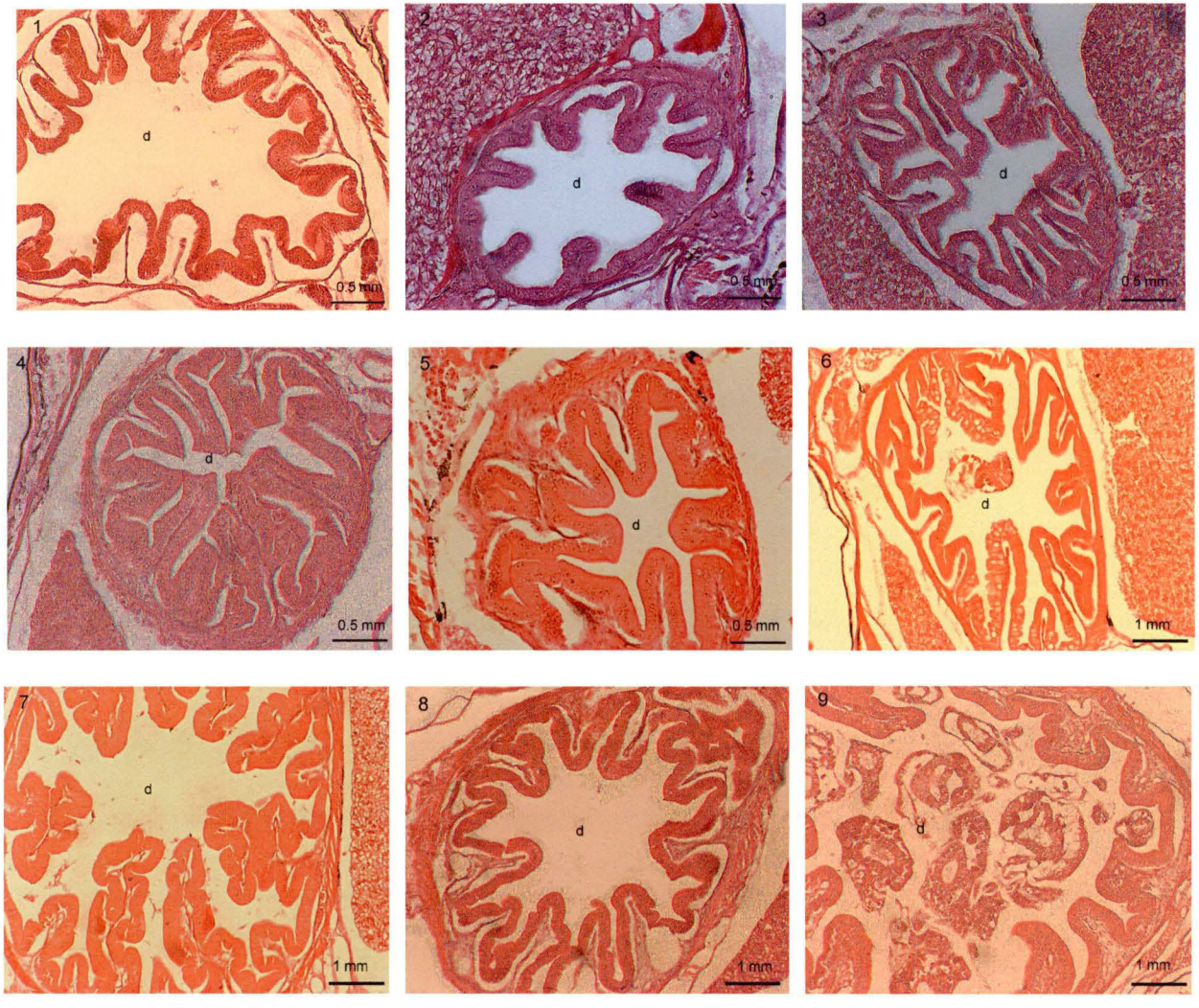
Figure 4.30. Transverse sections of the top region of the digestive tract stained with Haemotoxylin and Eosin. (1) The digestive tract from a newborn seahorse. (2) The digestive tract from a 1 day old seahorse. (3) The digestive tract from a 5 day old seahorse. (4) The digestive tract from a 7 day old seahorse. (5) The digestive tract from a 14 day old seahorse. (6) The digestive tract from a 21 day old seahorse. (7) The digestive tract from a 35 day old seahorse. (8) The digestive tract from a 49 day old seahorse. (9) The digestive tract from a 56 day old seahorse. Legend: (d) anterior digestive tract.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4.3.4. HISTOCHEMISTRY

By identifying the location of proteins in seahorses it can be suggested that gut enzymes are provided by the pancreas, clusters of mucus secreting cells within the intestinal wall, and what appear to be gastric cells (Figure 4.25) in the caudal region of the oesophagus.

4.4 DISCUSSION

Newborn seahorses are released from the pouch with what appear to be functional eyes due to strong pigmentation and their mouth and anus are open; they have a simple, straight digestive tract with epithelial lining; the pancreas and liver are already formed and what appear to be gastric cells are present in the caudal region of the oesophagus. These features suggest that the digestive tract of seahorses are equipped to assimilate food from the day of release from the male's pouch. There are very few changes in the seahorse digestive tract in older juveniles compared with newborns with the following exceptions. The intestinal valve began to develop on day 5 and became fully differentiated by day 7. The intestine started to loop around itself between 21 and 35 days after release. The mean gut epithelium becoming denser and more complex with age and this would allow for an increase in surface area available for enzyme secretion and hydrolysis of nutrients. Thus it could be said that seahorses are released with a near fully functioning digestive system.

Development of the digestive system of seahorses is quite different to the sequence of development described for many other fish species. While seahorses are released with the ability to feed, swim and catch prey, many fish species hatch as larvae rather then young juveniles and are without a functional gut, mouth and anus and must subsist on a parental derived yolk sac until the digestive and feeding systems are developed sufficiently for prey capture and digestion (Govoni et al., 1986; Green & McCormick, 2001). In anemone fish (*Amphiprion melanopus*), which is one of the fastest developing fish (Bellwood,

2000; Fisher et al., 2000) the alimentary tract is able to assimilate food (first feed on day one) well before the yolk sac is completely absorbed (day 3 after hatch) as they hatch with a digestive tract that is already differentiated into a foregut, midgut, and hindgut region and the liver and pancreas are partly formed. By day four the liver and pancreas have expanded rapidly and contain vacuoles, indicating they are functional areas of lipid and glycogen storage and the stomach, which increases rapidly in size and is fully differentiated midway through the larval period (Green & McCormick, 2001). Other fish such as turbot (*Scophthalmus maximus*) (Bisbal & Bengston, 1995); European sea bass (*Dicentrarchus labrax*), dover (*Microstomus pacificus*) and senegal sole (*Solea senegalensis*) (Boulhic & Gabaudan, 1992; Ribeiro et al., 1989), gilthead seabream (*Sparus aurata*), spotted sand bass (*Paralabrax maculatofasciatus*) and yellowtail flounder (*Limanda ferruginea*) (Baglole et al., 1997) hatch with a digestive tract that is more or less an undifferentiated straight tube. In the case of spotted sand bass first feeding on exogenous sources occurs two days after hatch when the mouth and anus open and at this time the yolk sac is only partially depleted. The digestive tract fully differentiates into a buccopharynx, oesophagus, stomach, anterior intestine, posterior intestine and rectum on day five and the liver and pancreas are found to have differentiated and function early in development as is the case in many species (Bisbal & Bengston, 1995; Guyot et al., 1995). Vacuoles, which aid in digestion, are found in the gut epithelium and these decrease in number with the appearance of gastric glands in the stomach. By day 16 the digestive tract of spotted sand bass are considered fully developed as the glandular stomach and pyloric caeca are fully differentiated (Pena et al., 2003). Unlike the spotted sand bass, which feeds on exogenous sources before the digestive tract is developed, flatfish species only begin to feed when the digestive tract is developed. For example, halibut only start feeding three days after hatch when the digestive tract has differentiated into a buccopharynx, oesophagus, pre-and post-valvular intestine and rectum. The appearance of functional goblet cells and gastric glands in flatfish have been

found to vary and these differences in differentiation and functionality could be due to rearing temperatures and/or feeding practices (Barnabe, 1994; Gisbert et al., 2004; Kuzmina & Gelman, 1998).

The development of the digestive tract of seahorses is also different from the development found in other carnivorous stomachless fish. For example, in the development of cyprinids that have a larval period of 35 days, the mouth opens on the third day after hatch and feeding on exogenous sources begins on day six when the digestive tract differentiates into the buccal cavity, pharynx, oesophageal swelling (which takes the place of the stomach), anterior and posterior intestine (Gordon & Hecht, 2002).

Digestion in fish starts in the mouth with mechanical digestion and chemical digestion of starches by amylase, which is secreted by the salivary glands (Bond, 1996; Mittal et al., 2002). The stomach further breaks down the food mechanically and proteins are initially broken down by gastric juice containing hydrochloric acid and pepsinogen, which is released from the gastric cells (Bond, 1996; Domenghini et al., 1998; Walford & Lam, 1993). The food (chyme) is then passed into the intestine, which is the site of complete digestion and absorption. In the intestine, food is chemically broken down by pancreatic juice and bile secreted into the lumen through the common bile duct and by enzymes such as disaccharides, aminopeptidase, lipase, intestinal amylase, dipeptidase and nucleotidase which are supplied by the intestinal membrane (Green & McCormick, 2001). Pancreatic juice contains sodium bicarbonate, which neutralises the acidity of the chyme, and enzymes such as trypsin and chymotrypsin that break down proteins, carboxypeptidase that breaks down amino acids, amylase that continues the break down of polysaccharides and lipase that hydrolyses triglycerides (fats). The bile contains bile salts which breakdown phospholipids and emulsify fats to enable them to be broken down

(Bond, 1996; Govoni et al., 1986; Hofer, 1991; Kapoor et al., 1975; Magnuson, 1969).

The stomach acts to store food and initiate digestion by mixing the ingested food particles with gastric juices. In fish that lack a stomach such as hagfish, lungfish (Dipnoi), minnows (Cyprinidae), wrasse (Labridae), Syngnathidae and some members of the Mugilidae, Cichlidae, Scaridae and Bleniidae families it is thought that protein digestion is poor because they lack a stomach and hence gastric cells which are responsible for protein digestion (Bond, 1996; Unal et al., 2001). However it has been found that some of these fish do have a gastric function because gastric cells have been found in their digestive tracts, for example pipe-fish, sculpins (Cottidae) and the grey mullet (*Mugil* sp.) (Fange & Groove, 1979; Osman et al., 1998). In the sculpins and grey mullet the oesophagus, which generally consists of stratified squamous cells and goblet cells, differs from other fish in that the caudal region consists of columnar epithelium cells without goblet cells and it is within this region that gastric cells have been found (Osman et al., 1998; Hussaini, 1947).

In this study on the pot-bellied seahorse it was found that the mucosa of the oesophagus consisted of stratified squamous cells and numerous goblet cells and then in the caudal region there was an abrupt change in cell type with the mucosa consisting of columnar epithelium cells, goblet cells were absent and what appear to be gastric cells were present. If this is the case, it could be said that seahorses do maintain a gastric function. It can also be suggested that, by location of proteins, seahorses produce enzymes in the pancreas and in mucus secreting cell clusters within the intestinal epithelium. Therefore, as seahorses appear to have gastric cells and pancreatic and intestinal enzymes, it could be said that their process of digestion is quite similar to the digestion of most other marine fish.

In conclusion it can be said that, unlike most other marine fish, pot-bellied seahorses are born as young juveniles with a near fully developed digestive tract which is comprised of a mouth, buccal cavity, pharynx, oesophagus, intestine and rectum and their major organs include a heart, gas bladder, liver, gall bladder, kidney and pancreas, all of which are formed prior to release. This study also suggests that although seahorses lack a stomach their digestion maybe similar to most other carnivorous marine fish, as seahorses appear to have a gastric function and gut enzymes are provided by the pancreas and the intestinal mucosa. Based on the development of the digestive tract it may be possible to feed seahorses an artificial diet from the day of release. This is however a preliminary study and further work on histological development, enzyme histochemistry, the classification of secretory products elaborated by glands and other mucus secreting cells is required to determine the ontogenetic functionality of the various regions of the digestive tract of pot-bellied seahorses.

CHAPTER FIVE

ONTOGENY OF THE GUT ENZYMES OF THE POT-BELLIED SEAHORSE

5.1. INTRODUCTION

Marine fish larvae are dependent upon live feeds, which are expensive to produce and manage. The nutritional quality in their diet often varies and sources of live feed can be highly unreliable (Kolkovski et al., 2000; Lazo et al., 2000; Nolting et al., 1999). A diet which has a high nutritional quality and is reasonably cost effective is required for the successful rearing of fish. One solution would be the use of an artificial diet which offers nutritional consistency, considerable savings in production costs and infrastructure, and has 'off the shelf' convenience (Kolkovski et al., 2000). Attempts to reduce the dependence upon live prey in fish culture has brought about intensive research on the development of artificial weaning diets. Whilst improvements have been reported with cofeeding live and dry diets, total replacement of live prey with an artificial diet has had limited success because artificial diets do not support the level of larval growth and survival found in larvae fed live diets (Kolkovski et al., 2000; Lazo et al., 2000; Murray et al., 1999; Nolting et al., 1999).

Factors, which may affect feed intake and be responsible for the limited success of artificial diets include presentation of the food, movement, attractants and ability of fish to digest certain dietary components. One of the main reasons for limited success with artificial diets is improper diet formulation. To determine specific dietary requirements of larval fish the ontogenetic development and physiological changes of the larval and juvenile digestive systems must be considered (Kolkovski et al., 2000; Lazo et al., 2000; Lundstedt et al., 1999ab; Nolting et al., 1999; Murray et al., 1999). During ontogenesis of fish larvae there are a number of important changes in the digestive tract (Kolkovski et al., 2000). Generally at hatching, fish larvae have a rudimentary gut composed of a straight undifferentiated tube that is closed at the mouth. The tube remains unchanged from mouth opening until completion of the yolk sac stage when it then undergoes rapid structural changes and is separated into a buccopharynx, foregut, midgut and hindgut. The extramural organs (liver and pancreas), which are formed at hatching,

become functional at first hatching and the stomach with gastric glands and pyloric caeca tends to develop just prior to metamorphosis (Cousin et al., 1987; Dabrowski, 1984; Govoni et al., 1986; Moyano et al., 1996; Person - LeRuyet, 1993).

Digestion is the process where ingested food is broken down to simple, small, absorbable molecules and occurs primarily by digestive enzymes (Guillame & Choubert, 1999; Lauff & Hofer, 1984; Uys & Hecht, 1987; Walford & Lam, 1993). Digestive enzymes are secreted into the lumen of the digestive tract and originate from the oesophagus, stomach, intestinal mucosa and from the pancreas (Baragi & Lovell, 1986; De Silva & Anderson, 1995; Verigina, 1991). There are two main categories of enzymes: 1) enzymes secreted by the pancreas and the stomach which are in the form of zymogens mixed in digestive juice, act on macromolecules, and are activated in the stomach and particularly in the top region of the intestine and 2) membrane bound enzymes which are intestinal and breakdown fragments of macromolecules (De Silva & Anderson, 1995; Guillaume & Choubert, 1999; Lauff & Hofer, 1984; Walford & Lam, 1993).

Enzymes are hydrolases, compounds capable of catalysing hydrolytic reactions (De Silva & Anderson, 1995; Guillaume & Choubert, 1999; Lauff & Hofer, 1984; Walford & Lam, 1993). Enzymes produced from zymogens include proteases, peptidases, glucosidases, lipases and nucleases. Proteases include a gastric enzyme pepsin and pancreatic enzymes: trypsin, chymotrypsin, elastase and collagenase, all of which hydrolyse internal bonds. Peptidases are represented by carboxypeptidases and carboxylesterase which hydrolyse external bonds and peptides respectively and originate from the pancreas. Glucosidases are represented by amylase, which originates from the pancreas and hydrolyses starch and by chitinase which may originate from the pancreas or stomach and is responsible for hydrolysing chitin. The lipases are represented by pancreatic lipase and esterases, which also originate from the pancreas and are responsible for the hydrolysis of triacylglycerols.

Nucleases represented by ribonuclease originates from the pancreas and is responsible for hydrolysis of nucleic acids (Baragi & Lovell, 1986; De Silva & Anderson, 1995; Guillaume & Choubert, 1999; Lauff & Hofer, 1984; Smith 1989; Walford & Lam, 1993).

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Membrane bound enzymes hydrolyse fragments of molecules formed by the action of zymogens and include other peptidases such as dipeptidyl peptidase and leucine aminopeptidase, other glucosidases such as maltase, other lipases and various other enzymes such as acid and alkaline phosphatase. Digestion is also aided by bile, which is made up of bile salts, organic anions and cholesterol. It is secreted from the liver and makes the intestinal medium alkaline and emulsifies lipids (Baragi & Lovell, 1986; De Silva & Anderson, 1995; Guillaume & Choubert, 1999; Lauff & Hofer, 1984; Smith 1989; Walford & Lam, 1993).

Generally at first feeding, the digestive system is functional though limited in terms of its capacity to process complex nutrients efficiently (Kim et al., 2001). Prior to stomach formation, which is the primary organ for digestive enzyme production, digestion of ingested food takes place within the lumen of the intestine and inside the luminal membrane of epithelial cells. The enzymes, which may be present at hatching or from the onset of feeding in larval fish, are secreted from the pancreas (Kim et al., 2001) and as fish larvae age, the morphological structure of the digestive tract generally becomes more complex. During fish ontogeny, patterns of enzyme activity vary and are subject to species-dependent differences (Timeyko & Novokov, 1987). In white sea bream (*Diplodus sargus*), for example, when the main enzymes involved in protein (acid and alkaline protease), carbohydrate (amylase) and lipid (lipase) digestion were studied it was found that amylase and lipase activity increased sharply during the first 3 days and then while amylase activity progressively decreased with age, lipase activity remained notably high until day 30 post-hatch when activity decreased (Cara et al., 2003). Alkaline protease activity showed an early appearance and peaked at 3 and 13

days post-hatch and again at 22 days post-hatch and then activity decreased to very low levels. Variation in the activity profiles of the digestive enzymes studied correlated either to developmental events such as the stomach becoming functional (day 22) or to a change in diet. Such sharp increases in lipase and amylase activity in the first three days were related to mouth opening, the peak in alkaline protease activity on day 13 correlated to a change in diet from rotifers to *Artemia* and the decrease in alkaline protease activity from day 22 onwards was due to the digestive tract being fully developed (Cara et al., 2003). Cuvier-Perez and Kestemont (2002) found that trypsin, chymotrypsin and amylase were present as early as hatching in the Eurasian perch (*Perca fluviatlis*) and that activities increased during the following days. Trypsin activity reached a maximum between day 4 and 7 and was followed by a sharp decrease until day 16 after which it remained constant. Chymotrypsin activity increased slowly during the first three weeks and then decreased slightly before remaining constant and amylase activity peaked on 9 and 21 days post-hatch. All intestinal enzymes (alkaline phosphatase, aminopeptidase and maltase) studied displayed a similar pattern with activity being detected from hatching onwards, peaking between day 16 and 23 and then remaining constant. Cuvier-Perez and Kestmont (2002) also found that pepsin activity was first detected on day 29 and this correlated with formation of the stomach.

In pre-feeding Pacific threadfin (*Polydactylus sexfilis*) and trevally (*Caranx melampygus*) it was found that while acid and alkaline amylase activities were undetectable, relatively high acid phosphatase and chitinase activities were present at hatching (Kim et al., 2001). At first-feeding activity of all enzymes tested increased with amylase activity increasing continuously in threadfin and increasing rapidly to nearly threefold in trevally. It was also found that lipase activity increased slowly to two-fold in 3.5 days in threadfin and in trevally at a much faster rate (Kim et al., 2001). Kim et al. (2001) also reported that activity of enzymes change with fish development and that enzyme activity tends to follow one of three profiles a) activity increases in

early larval development, b) activity increases and then remains fairly constant in later development or c) activity increases during the second half of development. In their study it was found that amylase activity increased during early development and then peaked midway between hatch and metamorphosis while the activity of protease and lipase was low during the first half of larval development and increased substantially during the second half of development.

It has also been noted that diet and ration affect (enhance, stop or delay) the type and activity of enzymes (Divakaran et al., 1999; Lemieux et al., 2003; Meton et al., 1999; Zambonino Infante & Cahu, 2001). Chan et al. (2004) suggested that the current understanding of digestive enzyme activity indicates a strong correlation with diet, that is herbivores possess higher levels of amylase than carnivores, and carnivorous fish have higher protease activities (eg pepsin and trypsin) than herbivorous fish. Sabapathy and Teo (1993) made a similar conclusion in their study showing that the digestive enzyme profiles of rabbitfish (*Siganus canaliculatus*) and sea bass (*Lates calcarifer*) correlated with their feeding habits. However, disparities have also been shown, as a number of carnivorous fish have been shown to have high levels of amylase on hatching. For example, seabream (*Sparus aurata*), which are pelagic marine carnivores have the ability to digest carbohydrates and it has been suggested that fish may rely more on carbohydrates than proteins in the first half of early development (Kim et al., 2001).

Digestive enzymes have been investigated as a means of understanding the nutritional needs of fish and the effect of dietary constituents on enzyme activity (Divakaran et al., 1999). The variations in digestive enzyme activity during larval development are indicative of the type of nutrients that fish can assimilate and digest (Eusebio et al., 2004). Understanding of the ontogeny of the digestive system is still rudimentary and a comprehensive analysis of ontogenetic changes occurring during the early life stages of fish is essential

for the development of optimal feeding strategies and for the formulation of artificial diets (Kumar et al., 2000).

Most marine fish on hatching are about 3 - 4 mm in length, have poor vision, small mouths and exogenous feeding is delayed (starting on about day 3). As fish get larger they undergo a series of morphological and physiological changes until metamorphosis into the juvenile stage occurs.

Pot-bellied seahorses are unusual in that they are born as juveniles (20 mm in length) and feed on exogenous food sources from day of release. There has been no reported work done on the ontogeny of seahorse gut enzymes and it could be inferred that their digestion may be similar to other stomachless fish as they are born and remain without a stomach or pyloric caeca.

Seahorses are pelagic, opportunistic feeders that feed on zooplankton and crustacean species such as mysids, krill, copepods, amphipods, euphausids and on small fish. In culture, newborn and early juvenile pot-bellied seahorses are fed instar II *Artemia*, later juveniles are fed instar II and adult *Artemia* and are weaned onto frozen mysids. The adult seahorse diet consists of adult *Artemia* and frozen mysids. To reduce the reliance on *Artemia* in the culture of the pot-bellied seahorse a better understanding of the seahorse's nutritional ability is required.

This study examines the digestive enzyme profiles of three selected enzymes in the pot-bellied seahorse (*Hippocampus abdominalis*) to determine the fishes digestive capacity, nutritional requirements and assess these changes as the seahorse ages. The three enzymes studied were trypsin, lipase and amylase to determine when seahorses were capable of digesting protein, lipids and carbohydrates respectively. In addition, the effects of diet and ration on enzyme activity were studied. A better understanding of the digestive capacity and nutritional requirements of pot-bellied seahorses will assist the development of a weaning sequence for juveniles and will ultimately

contribute to the development of artificial diets that meet the digestive capabilities of pot-bellied seahorses at various ages.

The general aim of this chapter is to determine the ontogenetic development of digestive enzymes of the pot-bellied seahorse and assess the effect of diet and ration on digestive enzyme activity. Specifically the study will take the following approaches:

a) To determine the enzyme activity of trypsin, lipase and amylase;
b) To determine if seahorses are released from the male's pouch with a full compliment of digestive enzymes;
c) To determine if diet and ration influence the enzymes of seahorses.

5.2. Materials and Methods

A cohort of 300 newborn seahorses from a single birth were transferred from Seahorse World Pty. Ltd. to the Aquaculture Centre. Seahorses were kept in a 50 L natural coloured fibreglass (fawn coloured sides) conico-cylindrical tank with a white gelcoat base within a recirculation system. The tank was cleaned daily and the seahorses were fed Algamac 3050™ enriched *Artemia* at a feed rate of 5% body weight day^{-1}.

At specific time intervals (Table 5.1) seahorses were collected for gut enzyme analyses. Seahorses were allowed to feed for 1 hour and then seahorses for gut enzyme analysis were euthanased in benzocaine and rinsed briefly in freshwater. The gut of each seahorse, which had been kept on ice, was then dissected out. The gill, heart, liver and gall bladder were removed and the gut was placed in an eppendorf tube, frozen in liquid nitrogen and stored in a -80°C freezer (to minimise enzyme degradation) for later analysis. Triplicate samples were taken at each time period and, depending on seahorse size, the number of seahorses per sample ranged between 1 and 10 (Table 5.1). This was to ensure that there was an adequate volume of homogenised seahorse gut

to determine the ontogenetic development of lipase, trypsin and amylase for the assays.

Table 5.1. The age when seahorses were sampled and the number of fish sampled at each sampling time.

Age at sampling (days)	Number of fish
1 (before feed)	10
1 (after feed)	10
3	10
7	10
14	10
21	6
28	4
35	4
42	4
49	3
56	3

Seahorses were also collected from the *Artemia* enrichment and ration experiment (Chapter 2) and the alternative live feed experiments (Chapter 3) to assess the effect of diet and ration on digestive enzyme activities. The biofouling trial consisted of 4 treatments: *Artemia* (control) fed fish, which were sampled in week 8 and biofouling fed fish, which were sampled in week 1, 4 and 8. The *Artemia* enrichment trial and ration trial consisted of fish fed with 6 different *Artemia* enrichments and 6 different daily feed rates respectively. Fish from these trials were sampled at the end of the two month feed trials.

All treatments: ontogeny (day 1, 3, 7,14, 21, 28, 35, 42, 49 and 56), biofouling (week 1, 4 & 8), *Artemia* enrichments (nil enrichment, Algamac 3050™,

Super Selco™, Protein Selco™, Artemac™ and Algae) and ration (1.25%, 2.5%, 3.75%, 5%, 6.25% and 7.5% BWd^{-1}) had 3 replicates and gut enzyme analyses were performed in duplicate (6 replicates per treatment) (see Appendix 9.11 for fish age details). Enzyme analysis of the prey items were also undertaken to assess the contribution of the prey to the digestive process.

5.2.1. Enzyme Analysis

Enzyme extraction

Frozen samples (gut) were thawed and homogenised in 1 ml of chilled 0.1 M Tris buffer (containing 0.02 M NaCl) at pH 7.5 for 1 minute using an electric (IKA - WERKE T8) Ultra-turrax homogeniser fitted with an S8N-8G dispersing tool. The homogenate was then centrifuged for 5 minutes at 10, 000 rpm to remove solids and the supernatant (enzyme extract) was aliquoted into eppendorf tubes and stored at
-20°C.

Enzyme assay

Enzyme assays were performed (in duplicate) by adding the enzyme specific buffer and substrate into a microplate (IWAKI 96 flat-bottom 200µl well microplate) and leaving it to incubate at the desired temperature for a few minutes. The enzyme was then added and the reaction was measured at the appropriate wavelength. Details of enzyme assays are enzyme specific and are discussed below.

A spectra Rainbow Thermo microplate reader operated by TECAN Magellan 2 software was used to record enzyme activity every 10 seconds for the duration of the assay (4 minutes). Readings from the spectrophotometer were converted to Au/min by Δabs / Δ time and enzyme activity was then measured as total activity (mg of enzyme in seahorse gut.seahorse^{-1}) by the following equation:

$$Au/min = €CL$$

Where C = the total enzyme activity, € = the extinction coefficient m^{-1} cm^{-1} and L = the light path (0.62). Units were defined as the concentration of enzyme to release 1μmol of substrate per minute and the amount of product formed was calculated using the enzyme specific molar extinction coefficient (see Appendix 9.12 for further information on calculations).

In this study activity of the enzyme was recorded as specific activity, which is defined as enzyme activity per mg of seahorse gut protein (ie enzyme activity expressed as a proportion of protein) rather than total enzyme activity (individual animal) due to the variability in weight, age and condition among seahorses. Protein concentration was determined by the method of Bradford (1977) using bovine serum albumin as the standard (Appendix 9.13).

Proteases

Trypsin was assayed using N-α-benzoylarginine-ρ-nitroanalide (BAPNA) dissolved in dimethylformamide (DMF) as the substrate. The final concentration of each assay was made up of 1.25 mM BAPNA in 200 mM Tris, and 200 mM NaCl (pH 7.5). The release of ρ-nitroanalide was measured at a temperature of 37°C, an absorbance of 405 nm and the molar extinction coefficient used under these conditions was 9, 300$M^{-1}.cm^{-1}$ (Stone et al., 1991).

Lipases

Lipase was assayed using 4-nitrophenyl caproate (4-NPC) in ethanol as the substrate. The final concentration of each assay was made up of 2.5 mM 4-NPC in 6 mM sodium taurocholate, 500 mM Tris (in HCl), and 100 mM NaCl (pH 8.5). The release of nitrophenol was measured at an absorbance of 405 nm and the molar extinction coefficient used under these conditions was 19, 800$M^{-1}.cm^{-1}$ (Gjellesvik et al., 1992).

Carbohydrases

Amylase activity was assayed using a Sigma micro-kit. The final concentration of each assay was made up of Infinity Amylase Reagent™ and enzyme extract. The enzyme activity was measured at a temperature of 37°C, an absorbance of 405 nm and the molar extinction coefficient used under these conditions was 10, 130$M^{-1}.cm^{-1}$.

In this study the enzyme activity of the diets was low in comparison to the enzyme activity levels produced by the seahorses, suggesting that it is unlikely that a substantial proportion of the enzyme activity measured in seahorses originated from the prey (Table 5.2.).

5.2.2. Statistical Analysis

All statistical analyses were performed using the SPSS software package (version 11.0 for windows). Mean values from duplicate assays were pooled and data was viewed for heterogeneity of variance using residual plots before carrying out one-way analysis of variance (ANOVA). Tukey's Honestly Significantly Different (HSD) post hoc test was used to identify differences among means.

Table 5.2. The amylase, lipase and trypsin activity of *Artemia* fed different *Artemia* enrichments diets, and the biofouling crustaceans.

	Amylase (mg of protein^{-1}.individual^{-1})	**Lipase** (mg of protein^{-1}.individual^{-1})	**Trypsin** (mg of protein^{-1}.individual^{-1})
Enrichment diets			
Super Selco	0.0022 ± 0.0001	0.0471 ± 0.0005	0.0011 ± 0.0001
Algae	0.0032 ± 0.0001	0.0084 ± 0.0021	0.0005 ± 0.0001
Artemac	0.0008 ± 0.0001	0.0497 ± 0.0007	0.0011 ± 0.0001
Unenriched	0.0032 ± 0.0001	0.0392 ± 0.0002	0.0007 ± 0.0001
Protein Selco	0.0024 ± 0.0001	0.0478 ± 0.0018	0.0016 ± 0.0001
Algamac	0.0008 ± 0.0001	0.0385 ± 0.0005	0.0012 ± 0.0001
Biofouling crustaceans			
Caprellids	0.0003 ± 0.0001	0.0482 ± 0.0008	0.0018 ± 0.0001
Hippomedon sp	0.0006 ± 0.0001	0.0311 ± 0.0071	0.0007 ± 0.0001
Biribus sp	0.0005 ± 0.0001	0.0321 ± 0.0041	0.0012 ± 0.0001

5.3. RESULTS

5.3.1. ENZYME ACTIVITY

Ontogeny

All seahorse age classes studied exhibited amylase, lipase and trypsin activity. Amylase specific activity was significantly different ($F = 41.914$, df 10, $p < 0.05$) across sample times. Activity increased from 0.089 ± 0.013 mg of protein^{-1}.seahorse^{-1} for newborn seahorses (before first feed) to 0.189 ± 0.01 mg of protein^{-1}.seahorse^{-1} in 3 day old seahorses and then dropped to 0.137 ± 0.01 mg of protein^{-1}.seahorse^{-1} in 7 day olds. Activity then increased to 0.207 ± 0.004 mg of protein^{-1}.seahorse^{-1} in 14 day olds and continued to increase slowly until seahorses were 28 days old and from this age onwards activity appeared to remain relatively constant (Figure 5.1a).

Lipase specific activity was significantly different ($F = 15.329$, df 10, $p < 0.05$) across sample times. Activity increased from 0.257 ± 0.023 mg of protein^{-1}.seahorse^{-1} for newborn seahorses (before feed) to 0.933 ± 0.069 mg of protein^{-1}.seahorse^{-1} for 35-day old seahorses. Lipase activity increased rapidly in the first 3 days and then continued to increase slowly until day 35 when activity appeared to become relatively constant (Figure 5.1b).

Trypsin specific activity was significantly different ($F = 70.611$, df 10, $p < 0.05$) across sample times. Activity increased slowly from 0.137 ± 0.011 mg of protein^{-1}.seahorse^{-1} for newborn seahorses (before first feed) to 0.284 ± 0.022 mg of protein^{-1}.seahorse^{-1} in 3 day olds; dropped to a level of 0.159 ± 0.06 mg of protein^{-1}.seahorse^{-1} in 7 day olds and then increased to 0.536 ± 0.084 mg of protein^{-1}.seahorse^{-1} in 14 day old seahorses. Trypsin activity continued to increase, at a faster rate than seen in the first 3 days, and reached a maximum activity level of 1.267 ± 0.03 mg of protein^{-1}.seahorse^{-1} in 35 day old seahorses (Figure 5.1c).

Effect of diet

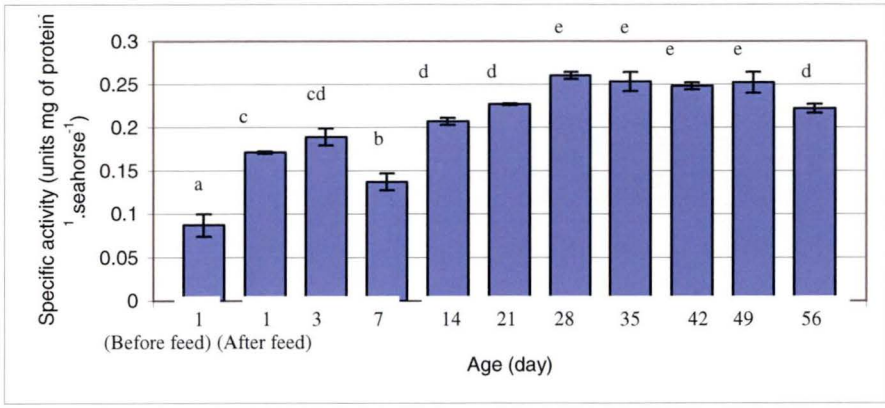
Different *Artemia* enrichments had no effect on the digestive enzymes of 19-week old seahorses. Amylase specific activity ranged from 0.208 ± 0.061 mg of protein^{-1}.seahorse^{-1} for Artemac to 0.329 ± 0.047 mg of protein^{-1}.seahorse^{-1} for Super Selco ($F = 0.953$, df 5, $p > 0.05$) (Figure 5.2a). Lipase specific activity ranged from 1.187 ± 0.174 mg of protein^{-1}.seahorse^{-1} for Super Selco to 1.359 ± 0.153 mg of protein^{-1}.seahorse^{-1} for Protein Selco ($F = 0.321$, df 5, $p > 0.05$) (Figure 5.2b). Trypsin activity ranged from 0.73 ± 0.065 mg of protein^{-1}.seahorse^{-1} for Super Selco to 1.161 ± 0.194 mg of protein^{-1}.seahorse^{-1} for Artemac ($F = 1.029$, df 5, $p > 0.05$) (Figure 5.2c).

When the activity of amylase was compared between seahorses fed biofouling and enriched *Artemia* it was found that there was no significant difference. When the activities of lipase and trypsin were compared between seahorses fed biofouling and enriched *Artemia* significant differences were found but Tukey's HSD test showed no difference between the dietary treatments for both lipase and trypsin.

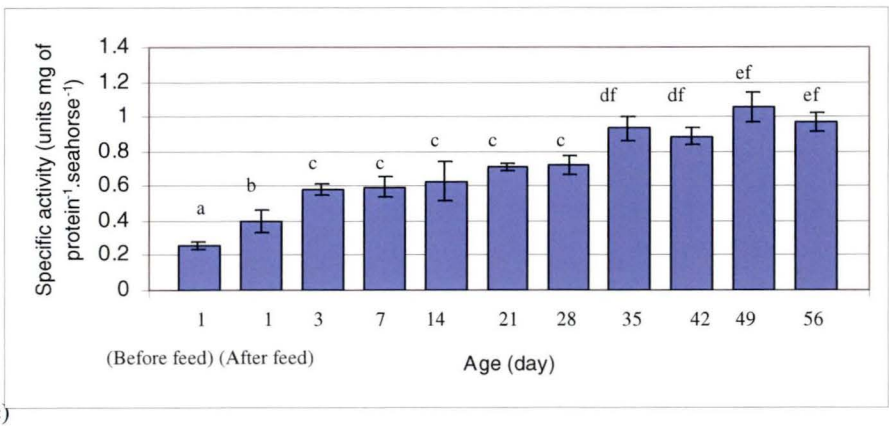
Amylase activity for biofouling fed fish was 0.218 ± 0.018 mg of protein^{-1}.seahorse^{-1} and 0.18 ± 0.029 mg of protein^{-1}.seahorse^{-1} for *Artemia* fed fish ($F = 1.217$, df 1, $p > 0.05$) (Figure 5.3a). Lipase activity was significantly ($F = 5.368$, df 1, $p < 0.05$) higher in biofouling fed fish (2.59 ± 0.289 mg of protein^{-1}.seahorse^{-1}) than *Artemia* fed fish (1.589 ± 0.321 mg of protein^{-1}.seahorse^{-1}) (Figure 5.3b). While trypsin activity was significantly ($F = 0.819$, df 1, $p < 0.05$) higher in *Artemia* fed fish (0.379 ± 0.106 mg of protein^{-1}.seahorse^{-1}) than biofouling fed fish (0.278 ± 0.034 mg of protein^{-1}.seahorse^{-1}) for biofouling fed fish (Figure 5.3c).

Figure 5.1. Ontogeny of specific enzyme activity from whole pot-bellied seahorse gut enzyme extracts. (a) Amylase activities are the mean (units.mg of protein^{-1}seahorse^{-1}) ± S.E where units are in nmol Ethylidene .min^{-1}, (b) Lipase activities are the mean (units.mg of protein^{-1}seahorse^{-1}) ±S.E where units are in nmol nitrophenol .min^{-1} and (c) Trypsin activities are the mean (units.mg of protein^{-1}seahorse^{-1}) ± S.E where units are in nmol p-nitroanalide.min^{-1}. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

a)


Specific activity (units mg of protein
-1.seahorse-1)
0.3
0.25
0.2
0.15
0.1
0.05
0
a
c
cd
b
d
d
e
e
e
e
d
1
1
3
7
14
21
28
35
42
49
56
(Before feed) (After feed)
Age (day)

b)


Specific activity (units mg of
protein-1.seahorse-1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
c
c
c
c
df
df
ef
ef
1
1
3
7
14
21
28
35
42
49
56
(Before feed) (After feed)
Age (day)

c)

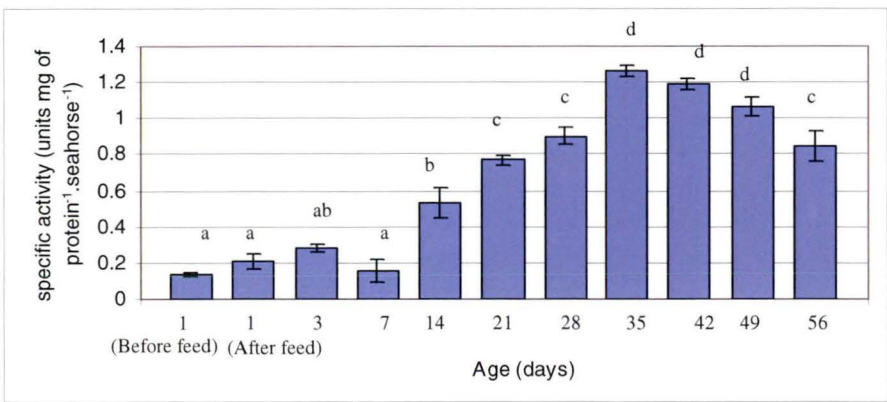
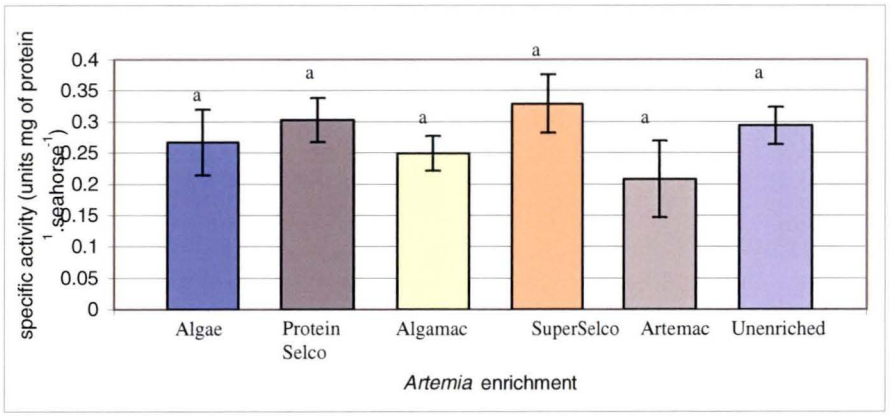
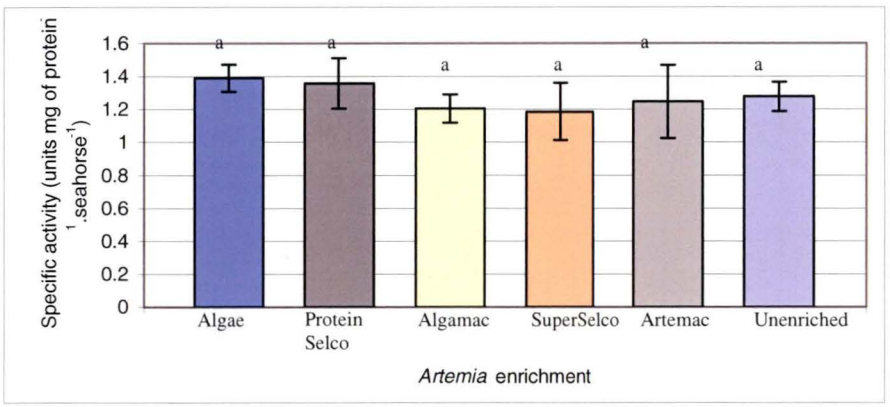

specific activity (units mg of
protein-1.seahorse-1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
ab
a
b
c
c
d
d
d
c
1
1
3
7
14
21
28
35
42
49
56
(Before feed) (After feed)
Age (days)

Figure 5.2. Specific enzyme activity of 19-week old pot-bellied seahorses fed *Artemia* enriched with different diets (mixed algae (*Chaetoceros*, *Isochrysis*, *Tetraselmis*), Protein Selco™, Algamac 3050™, Super Selco™, Artemac™ and an unenriched (control) at a feed rate of 5% body weight day^{-1}. (a) Amylase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol Ethylidene $.min^{-1}$, (b) Lipase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol nitrophenol $.min^{-1}$ and (c) Trypsin activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ±S.E where units are in nmol p-nitroanalide.min^{-1}. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

a)


specific activity (units mg of protein-1.seahorse-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
a
a
a
Algae
Protein Selco
Algamac
SuperSelco
Artemac
Unenriched
Artemia enrichment

b)


Specific activity (units mg of protein-1.seahorse-1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
Algae
Protein Selco
Algamac
SuperSelco
Artemac
Unenriched
Artemia enrichment

c)

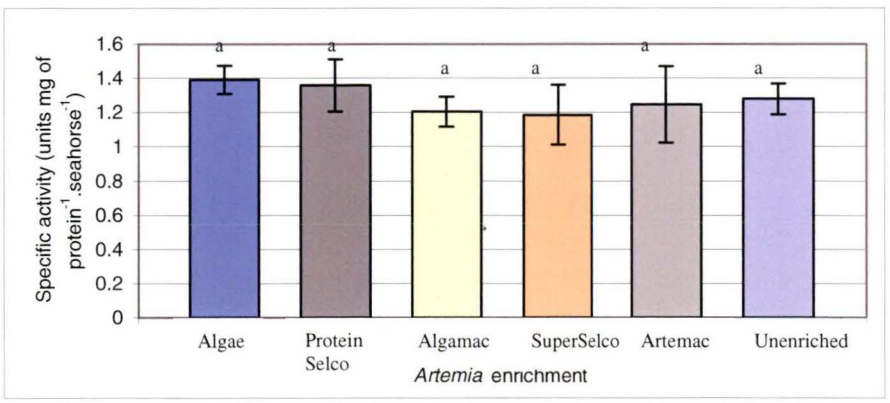
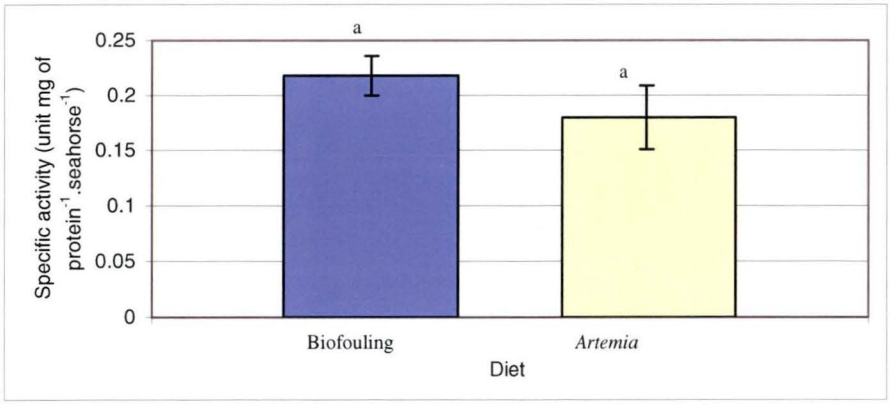
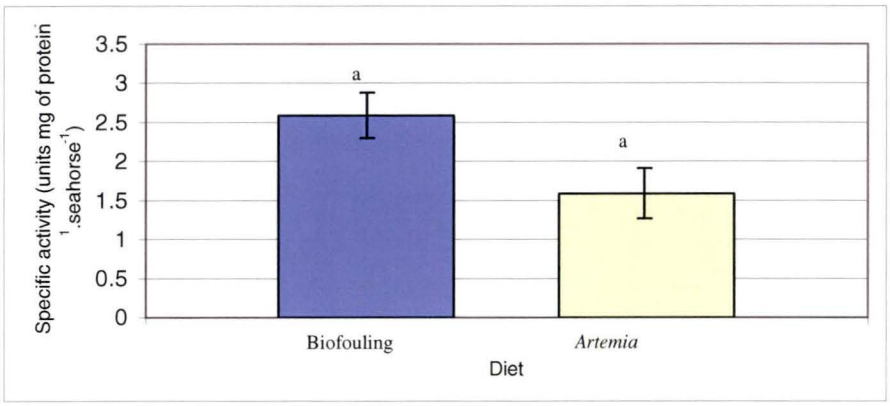

Specific activity (units mg of protein-1.seahorse-1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
Algae
Protein Selco
Algamac
SuperSelco
Artemac
Unenriched
Artemia enrichment

Figure 5.3. Specific enzyme activity of 21-week old pot-bellied seahorses fed biofouling crustaceans and *Artemia* enriched with Algamac-3050 at a feed rate of 10% body weight day^{-1}. a) Amylase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol Ethylidene $.min^{-1}$, b) Lipase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol nitrophenol $.min^{-1}$ and c) Trypsin activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ±S.E where units are in nmol p-nitroanalide.min^{-1}. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

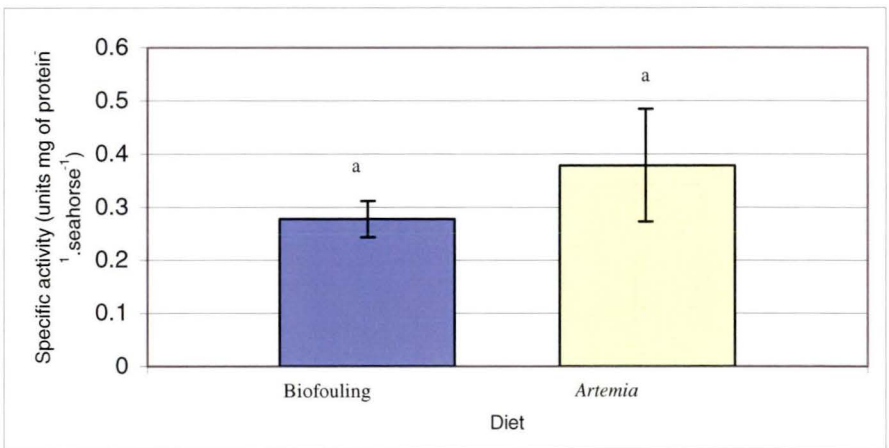
a)


Specific activity (unit mg of protein^{-1}.seahorse^{-1})
0.25
0.2
0.15
0.1
0.05
0
a
a
Biofouling
Artemia
Diet

b)


Specific activity (units mg of protein^{-1}.seahorse^{-1})
3.5
3
2.5
2
1.5
1
0.5
0
a
a
Biofouling
Artemia
Diet

c)


Specific activity (units mg of protein^{-1}.seahorse^{-1})
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
Biofouling
Artemia
Diet

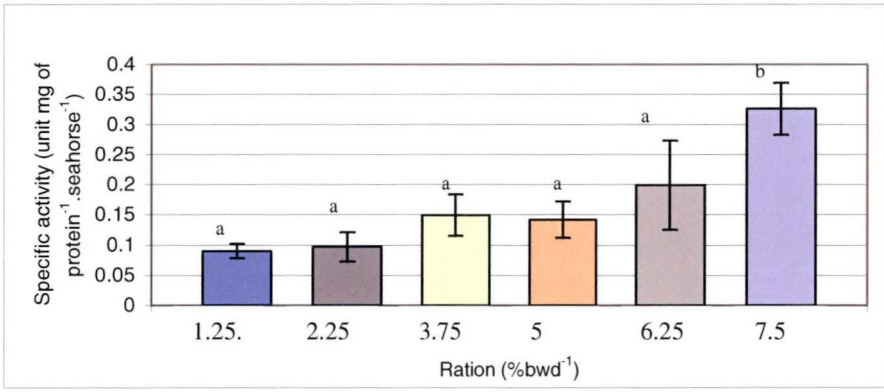
Effect of Ration

Ration had a significant effect on the activity of amylase, lipase and trypsin of 15- week old seahorses. Amylase activity increased with higher rations ($F = 4.363$, df 5, $p < 0.05$). Fish fed rations of 1.25% and 2.5% body weight day^{-1} had similar enzyme activity with a recorded level of 0.09 ± 0.012 mg of $protein^{-1}seahorse^{-1}$ and 0.097 ± 0.024 mg of $protein^{-1}seahorse^{-1}$ respectively. Activity level increased to 0.149 ± 0.030 mg of $protein^{-1}seahorse^{-1}$ when fish were fed a 3.75% body weight day^{-1} ration, 0.199 ± 0.074 mg of $protein^{-1}seahorse^{-1}$ when fed 6.25% body weight day^{-1} and up to 0.326 ± 0.043 mg of $protein^{-1}seahorse^{-1}$ when fed a ration of 7.5% body weight day^{-1} (Figure 5.4a).

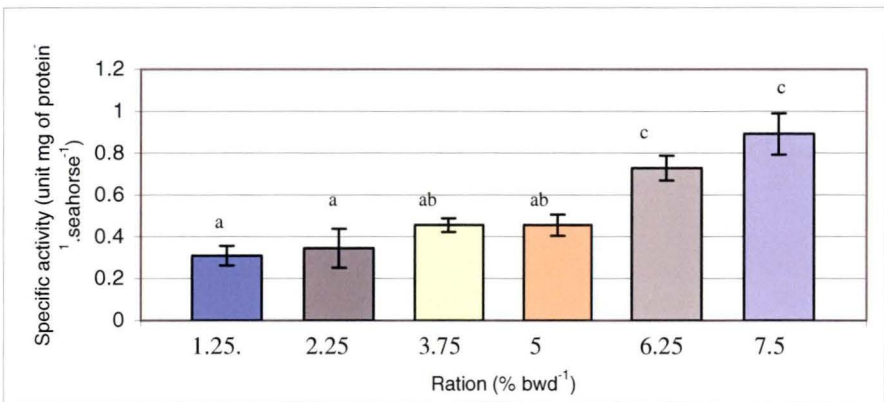
Lipase activity was high and exhibited the same pattern as amylase with activity increasing with higher rations ($F = 11.207$, df 5, $p < 0.05$). Lipase activity increased from 0.31 ± 0.048 mg of $protein^{-1}seahorse^{-1}$ at the 1.25% body weight day^{-1} ration to 0.892 ± 0.099 mg of $protein^{-1}seahorse^{-1}$ at the 7.5% body weight day^{-1} ration (Figure 5.4b). Trypsin activity also increased with ration sizes ($F = 3.724$, df 5, $p < 0.05$) with fish fed a 1.25% body weight day^{-1} ration and a 7.5% body weight day^{-1} having an activity level of 0.139 ± 0.024 mg of $protein^{-1}seahorse^{-1}$ and 0.417 ± 0.085 mg of $protein^{-1}seahorse^{-1}$ respectively (Figure 5.4c).

Figure 5.4. Specific enzyme activity of 15-week old pot-bellied seahorses fed different daily rations of *Artemia* enriched with Algamac-3050 (5% body weight day^{-1} was considered 100%). a) Amylase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol Ethylidene $.min^{-1}$, b) Lipase activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ± S.E where units are in nmol nitrophenol $.min^{-1}$ and c) Trypsin activities are the mean (units.mg of $protein^{-1}seahorse^{-1}$) ±S.E where units are in nmol p-nitroanalide.min^{-1}. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

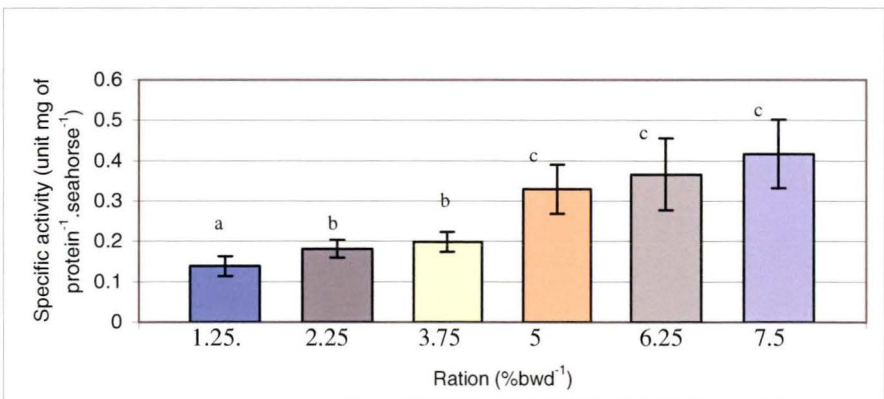
a)


Specific activity (unit mg of protein-1.seahorse-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
a
a
b
1.25.
2.25
3.75
5
6.25
7.5
Ration (%bwd-1)

b)


Specific activity (unit mg of protein-1.seahorse-1)
1.2
1
0.8
0.6
0.4
0.2
0
a
a
ab
ab
c
c
1.25.
2.25
3.75
5
6.25
7.5
Ration (% bwd-1)

c)


Specific activity (unit mg of protein-1.seahorse-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
b
c
c
c
1.25.
2.25
3.75
5
6.25
7.5
Ration (%bwd-1)

5.4. DISCUSSION

Of the digestive enzymes analysed (trypsin, lipase and amylase) all were detected at every age studied revealing that across this range newborn to 56 days old pot-bellied seahorses are capable of digesting protein, lipid and carbohydrate at all stages of development. Trypsin and lipase activities were greater than amylase activities indicating that seahorses rely more heavily on protein and lipid than carbohydrate for their early nutrition. Cultured seahorses may be fed instar II *Artemia*, adult *Artemia*, amphipods, krill and mysids, all of which generally have a high protein and lipid content and a low carbohydrate content (Nicholls et al., 1999). When considering the composition of the prey, the higher trypsin and lipase activities and the lower amylase activity it can be suggested that pot-bellied seahorses are almost exclusively carnivores but are able to utilise carbohydrates.

The presence of enzymes in newborn seahorses indicates that they are capable of digestion prior to the onset of feeding. This trend is also seen in other marine fish such as cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), bluefin trevally (*Caranx melampygus*), Pacific threadfin (*Polydactylus sexfilis*), sea bream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*) which were all capable of digesting protein and lipids from hatch onwards (Cara et al., 2003; Kim et al., 2001; Nolting et al., 1999; Perez-Casanova et al., 1999). It has been suggested that this presence of enzyme activity prior to first-feeding could be a general feature of fish larvae that hatch with large yolk sacs (Cuvier-Perez et al., 2002; Nolting et al., 1999).

An increase in specific activities of amylase, lipase and trypsin with age reflects an increasing digestive capacity during early development and this is required to ensure that the metabolic and structural requirements of larger seahorses are met. Changes in the activity of digestive enzymes as a general rule are associated with the age and morphological development of the digestive system (Cuvier-Peres et al., 2002). The intestine comprises a simple

columnar epithelium covered by a thin brush border, which is primarily concerned with the absorption of digested food (Kumar et al., 2000). On hatching, larval fish only possess a few small microvilli which limits the ability of fish to utilise certain feeds and dietary components. With age, the gut epithelium complexity increases (number and size of microvilli) and digestive organs become functional (Cara et al., 2003). As a consequence of the morphological changes to the digestive tract the capacity for digestion is enhanced. For example Kim et al. (2001) found that with age the magnitude for lipid and protein digestion increased and the capacity for carbohydrate digestion decreased as fish no longer required carbohydrates to fill the high need of protein between endogenous and exogenous feeding. Cara et al. (2003) noted that the gastric glands of white sea bream (*Diplodus sargus*) appeared around 20 days post-hatch, the stomach became fully functional by 30 days post-hatch and a sharp increase in the activity of most of the enzymes was observed at 22 days post-hatch. This peak in enzyme activity was probably due to an increase in supply of suitable substrates for other digestive enzymes.

In this study trypsin activity was present in newborn seahorses. There was little to no change in activity for the first 2 weeks, activity then increased at a faster rate, peaked on day 35, and then activity started to decline slowly. This trend in activity was different to other marine fish for example Nolting et al. (1999) found that trypsin activity in the European sea bass increased rapidly until day 7 and then at a slower rate until day 17 after which there was little to no change in activity. Cuvier-Perez et al. (2002) found that trypsin activity in the European perch (*Perca fluviatilis*) was present as early hatching and increased immediately with maximum activity occurring between days 4 and 7. Activity then decreased sharply and from day 16 remained constant. The initial increase in trypsin activity observed in sea bass and perch demonstrates the existence of proteolytic digestive potential that allows digestion of the first exogenous feeds and it has been suggested that initially there could be a correlation between trypsin activity and age (Lemieux et al., 2003; Nolting et

al., 1999). From this study it may be said that pot-bellied seahorses are capable of protein digestion from release, however their enzyme profile lacks the rapid initial increase in trypsin activity seen in other fish and this could be due to seahorses utilising different dietary components such as fats or carbohydrates during early development. Or because seahorses are released as an early juvenile and therefore the rapid rise seen in other marine larval fish occurs during development within the pouch.

Lipase activity was present in newborn seahorses and continued to increase slowly with age. A similar trend was observed by Kim et al. (2001) as the lipase activity in threadfin (*Polydactylus sexfilis*) and blue fin trevally (*Caranx melamoygus*) increased from first feed onwards. When Lazo et al. (2000) studied the activity profile for lipase in red drum (*Sciaenops ocellatus*) lipase activity was detected at hatching, found to increase sharply just prior to first feeding and thereafter activity continued to increase as a function of age and length. Cara et al. (2003) on the other hand found that lipase activity of white sea bream (*Diplodus Sargus*) remained more or less constant from day three onwards and that the enzyme profile obtained showed that enzyme activity was affected by major events such as the start of stomach functionality (3 days post hatch) or weaning. In relation to seahorses this suggests that the increase in lipase activity over time is a function of seahorse age, in that activity could be related to the development of the gut. For example Eusebio et al. (2004) found that lipase activity in the grouper (*Epinephelus coioides*) gradually increased and that it was related to the development of the pyloric caeca and the intestine. It was also noted that fish larvae were capable of lipid digestion from the onset of feeding and lipase secretion seems to be induced by food intake (Cara et al., 2003; Kim et al., 2001), which will be discussed later.

Although activity levels were much lower than the trypsin and lipase activities, amylase activity was present in newborn seahorses. Activity significantly increased after first feeding and continued to increase until day

14 when the activity became more or less constant. Perez-Casanova et al. (1999) found that Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) showed no amylase activity from hatch onwards and suggested that this is not unusual as they are carnivores which feed on zooplankton in their early life stages. Lemieux et al. (2003) found that amylase activity was present in the Arctic charr (*Salvelinus alpinus*) but decreased with age and stated that this was a normal maturation process of the pancreas in young fish. Kim et al. (2001) found that amylase activity was undetectable at hatch in the sea bream but increased prior to first feeding and continued to increase until midway between hatching and metamorphosis. Cuvier-Perez et al. (2002) found that amylase activity exhibited two maxima, one at day 9 and the other at day 23. Presence of amylase in marine fish prior to first feeding suggests that this enzyme may be important in the early development of fish and may be because carnivorous fish rely on dietary carbohydrates to fill the protein demand during the changeover between endogenous and exogenous feeding (Kim et al., 2001; Murray et al., 1999; Sebapathy & Teo, 1993).

The presence of amylase activity in young seahorses also suggests that they have the capacity to digest the chitinous exoskeletons of crustaceans such as copepods, amphipods and mysids, which are abundant potential prey items in the wild.

Activities of enzymes are also affected by changes in the quantity and composition of available food (Cara et al., 2003; Meton et al., 1999). Some studies show a strong relationship between diet and digestive enzymes (Kuzmina, 1996) while others show that diet has little to no effect on the activity of enzymes.

In the present study it was found that different diets fed to seahorses (*Artemia* enriched with different enrichments and biofouling crustaceans) had little or no effect on the activity of enzymes but activity increased significantly with

increasing ration size. Kumar et al. (2000) found no significant difference in enzyme activity between larvae fed live plankton and refrigerated plankton until day 9 and from day 10 onwards activity was significantly higher in fish fed live plankton. Lazo et al. (2000) found that larvae fed live food had significantly higher tryptic activity than those fed a compound diet until day 27 when activity became similar.

The lack of change in enzyme activity should be expected as it would be assumed that the digestive tract of 19 and 21 week old seahorses fed *Artemia* enriched with different diets and biofouling crustaceans respectively is fully developed and at its maximum metabolic capacity at this time. It was found in previous work on the histological development of the digestive tract (chapter 4) that seahorses are released with a near fully developed digestive tract. The main differences found overtime included the development of an intestinal valve separating the rectum from the intestine on day 7, the gut started to loop around itself between day 21 and 35 and the gut epithelium became thicker with age. It was also noted that seahorses remain stomachless and what appear to be gastric cells, which are located in the stomach of other fish, were found in the oesophagus. The increase in enzyme activity with increasing ration sizes was expected; as stated earlier enzyme secretion is induced by food intake and generally ration size is highly correlated with growth, which brings about normal development and as a consequence increased substrates for digestive enzymes (Cara et al., 2003; Kim et al., 2001; Lazo et al., 2000; Meton et al., 1999).

In this study it was found that when comparing specific enzyme activity across the experiments, activity levels varied. For example, in the *Artemia* enrichment trial seahorses fed Algamac at a feed rate of 5% body weight day^{-1} had a specific amylase activity of 0.35 U mg $protein^{-1}$ while seahorses in the ration trial which were also fed *Artemia* enriched with Algamac and at a feed rate of 5% body weight day^{-1} had a specific amylase activity of 0.15 U mg $protein^{-1}$. The difference in specific activity may have been due to the age of

the fish as seahorses in the *Artemia* enrichment and ration trial were 15 and 21 weeks old respectively. Although the ontogenetic study only looked at seahorses up to 56 days (8 weeks) old and it was found that enzyme activity tended to become relatively constant from 28 to 35 days onwards activity may change in older seahorses. Further work on the ontogenetic development of enzymes would be needed to determine this. It was also found that seahorses in the biofouling trial, which were fed *Artemia* enriched with Algamac at a feed rate of 10% body weight day^{-1}, had a specific amylase activity of 0.21 U mg $protein^{-1}$. This difference in specific activity may have been due to the advanced age of seahorses (21 weeks old) or the higher ration level.

Another finding in this study was that fish fed biofouling had higher activities of amylase and lipase than *Artemia* fed fish and *Artemia* gave rise to higher trypsin activity. A significant difference was found between the lipase and trypsin activity of fish fed biofouling and *Artemia* but Tukey's HSD post hoc test showed no difference between treatments possibly as a result of variation within the data. It was also found in this study that lipase, trypsin and amylase activity of seahorses fed *Artemia* enriched with different diets varied suggesting that the composition of diets may differ but again variation within the data may have masked any differences. This is a baseline study and further research is required to detail the digestive enzymes of seahorses and their profiles and determine whether diet influences enzyme activity.

Other studies have shown that the enzyme profiles of fish are affected by their development (eg first feeding and metamorphosis) and change in diet. Although seahorses are unlike other marine fish, in that they are released from the pouch as early stage juveniles with a near complete digestive tract, development may well affect their enzyme profiles. That is in this study amylase and lipase activity significantly increased before and after feed on the day of release, which may correlate to first feeding and amylase, trypsin and lipase activity showed a trend to decrease on day 7 which may correlate to the development of the intestino-rectal valve. It was also noted that amylase

activity reached its peak on day 28 with little or no further change in activity, lipase activity which increased slowly from release became more constant from day 35 onwards and trypsin activity reached its peak on day 35 and then decreased slowly. This trend for enzyme activity to peak then become constant between days 28 and 35 may correlate to the gut starting to loop around itself and the intestinal mucosa becoming thicker. During further work on the histological development of seahorses it was noted that what appear to be gastric glands, which are located in the stomach of other fish, were found in the oesophagus. Given that it could be said that seahorses are capable of gastric function it would have been ideal to determine the protein digestion capabilities of different aged seahorses. Due to gut enzyme work being completed before the work on the histological development of seahorses was finalized pepsin activity was not analysed in this study.

In conclusion, pot-bellied seahorses (*Hippocampus abdominalis*) are released from the pouch as newborns with the digestive enzymes trypsin, lipase and amylase, which initially increase with age and then become constant. This diverse enzyme profile, which is present from release onwards, enables seahorses to utilise many different dietary components as sources of energy and materials for growth and is consistent with randomly encountering a wide variety of potential prey. In this study diet did not affect the levels of enzyme activity and this could be because the composition of the prey species looked at were not sufficiently different to affect the enzyme profiles of seahorses, the variation within the data set was too large to show a significant difference between diets or due to the improved digestive capabilities of older seahorses. It is possible that diet may have more of an effect on the gut enzymes of younger seahorses.

The intestinal enzymes are efficient from early developmental stages suggesting that a compound diet could be introduced into the feeding sequence of early juvenile seahorses. However, as this is one of the first studies on the ontogeny of gut enzymes and the affect of diet on enzyme

profiles of pot-bellied seahorses, a more comprehensive analysis of the seahorse gut enzyme profile is required as well as further research on their ontogenetic development, physiological changes and feeding behaviour before an artificial diet can be tailored to meet the nutritional requirements and digestive capabilities of pot-bellied seahorses.

CHAPTER SIX

COST BENEFIT ANALYSIS

6.1. INTRODUCTION

The effect of *Artemia* on the growth of pot-bellied seahorses (chapter 2) and the effect of alternative diets (copepods, biofouling crustaceans, frozen amphipods and frozen mysids) (chapter 3) on their growth have been studied to find diets to replace or reduce the reliance on *Artemia* in seahorse culture.

The studies showed that the growth of seahorses fed alternative feeds was comparable to the growth of seahorses (same age) fed enriched *Artemia,* suggesting it would be feasible to replace expensive *Artemia* with alternative diets.

While it has been shown that *Artemia* may be reduced or replaced it is unknown if such a replacement would produce any financial benefit. In this chapter, an analysis of the projected costs and benefits of alternative diets and *Artemia* will be conducted. The cost of producing 1,000 and 10,000 seahorses will be determined.

6.2. COST BENEFIT ANALYSIS

These studies have been carried out under associated sections to ascertain the comparable costs of producing 1,000 and 10,000 seahorses fed different diets (*Artemia*, copepods, biofouling crustaceans, amphipods and mysids).

In such an analysis there could be numerous combinations of parameters used within production. This outline will attempt to standardise many background costs to highlight relative costs between diets. In the future data could be manipulated further by the use of such approaches as sensitivity analysis (for example).

The study is set out as follows:

6.2.1. Issues associated with culturing or collection of diets and cost of seahorse production fed alternative diets

a) *Artemia*

- Issues associated with the use of *Artemia*

- Feed preparation
- Costs involved with culture
- Projected costs of producing 1,000 and 10,000 seahorses

b) Copepods

- Issues associated with the use of copepods
- Feed preparation
- Costs involved with collection
- Projected costs of producing 1,000 and 10,000 seahorses

c) Biofouling composition

- Issues associated with the use of Biofouling crustaceans
- Feed preparation
- Costs involved with collection
- Projected costs of producing 1,000 and 10,000 seahorses

d) Amphipods

- Issues associated with the use of amphipods
- Feed preparation
- Costs involved with culture
- Projected costs of producing 1,000 and 10,000 seahorses

e) Mysids

- Issues associated with the use of mysids
- Analysis of costs
- Projected costs of producing 1,000 and 10,000 seahorses

6.2.2. Summary of projected costs of producing 1,000 and 10,000 seahorses fed different diets.

6.2.3. Forecast cost of producing seahorses to market size fed a number of feeding regimes

6.2.1. ISSUES ASSOCIATED WITH CULTURING OR COLLECTION OF DIETS AND COST OF SEAHORSE PRODUCTION FED ALTERNATIVE DIETS

a) *ARTEMIA*

ISSUES ASSOCIATED WITH THE USE OF *ARTEMIA*

Benefits

- *Artemia* are good for nutritional enhancement - bioencapsulation;
- *Artemia* cysts are convenient in that they can be stored for years (1-2);
- Grow out to range of sizes (eg instar I, instar II and adult);
- Culture of instar II *Artemia* can be achieved in 36 hours;
- Enrichment diets and products available to minimise bacterial loads.

Disadvantages

- Tanks are required for hatching and ongrowing *Artemia*;
- Culture requires a temperature controlled room;
- Purchase of an expensive food source (*Artemia* cysts);
- Purchase of expensive enrichment diets;
- Time consuming process especially grow-out;
- Potential for expensive culture crashes.

COSTS INVOLVED WITH CULTURE

The costs of *Artemia* include not only the purchase of cysts and enrichment diets but also specific culture facilities, maintenance costs and running costs. The purchase price of cysts (Table 6.1) and enrichment diets (Table 6.2) vary with market supply and demand. Prices in this study are based on figures from Primo Aquaculture August 2005 (www.primo.nt.au).

Facility costs include a temperature controlled room, hatching bags, tanks for ongrowing, high watt fluorescent lights, a fresh and salt water supply and a reservoir for acclimating water. Maintenance costs include: hatching bags, tank fittings, pumps and hoses, airline and light tubes and running costs include: electricity, water and labour.

Table 6.1. The purchase price of *Artemia* cysts from Primo Aquaculture August 2005.

Product	Hatch rate	Weight	Price per unit (A$)
INVE GSL *Artemia* AAA	90%	425 g	69.50
INVE GSL *Artemia* AA	85%	425 g	59.50
INVE GSL *Artemia* A	80%	425 g	45.50
Neptune GSL *Artemia*	88%	425 g	59.50

Table 6.2. The purchase price of *Artemia* enrichments from Primo Aquaculture and *Artemia* international, August 2005.

Product	Weight	Feed type	Price per unit US$
Primo Aquaculture			
INVE DC Super Selco	1 kg bottle	emulsion	223.00
INVE DHA Selco	1 kg bottle	emulsion	129.50
INVE DC DHA Selco	1 kg bottle	emulsion	137.00
INVE DHA Protein Selco	1 kg bottle	emulsion	218.00
Artemia International			
Selco	1 kg bottle	emulsion	120.00
Super Selco	1 kg bottle	emulsion	185.00
Algamac 2000	500 g bag	emulsion	205.00
Algamac 3050	500 g bag	emulsion	230.00

FEED PREPARATION

This section although not vital in a cost benefit analysis has been added to show what activities contribute to the costs of the feeds.

The hatching and enrichment process includes:

Hatching *Artemia*

- cysts hydrated at a density of 2 gL^{-1} in 25°C tap water for 1 hour.
- hydrated cysts in 12.5% sodium hypochlorite for 10 minutes.
- decapsulated cysts collected, sieved and washed.
- washed cysts suspended in 6 L of 25°C seawater.
- 24 h after decapsulation hatched nauplii harvested and washed with freshwater.
- nauplii resuspended in acclimated seawater for ongrowing.

Enrichment

- instar II *Artemia* harvested and enriched at required dose rate.
- enrichment dose rates: Selco 0.3 g/L, SuperSelco 0.6g/L and Algamac 3050 0.2g/L. Enriched at a density of 100, 000 nauplii/L.
- *Artemia* left to enrich over night (15 h).
- *Artemia* are harvested, washed with freshwater and returned to seawater.
- feed is removed to be fed out to seahorses and remainder is re-enriched for afternoon meals.

PROJECTED COST OF PRODUCING *ARTEMIA*

The criteria listed below have been used in this study

- *Artemia* cysts used are Argentemia gold.
- 290,000 cysts per gram and an 80% hatch rate is achieved.
- Enrichment used is Algamac 3050 (dose rate 0.2g/L).
- *Artemia* is fed out over 2 meals. The afternoon meal is re-enriched.

For the purpose of this study the facility and maintenance costs have been excluded.

Table 6.3. Projected cost of producing instar II *Artemia* in 6 L hatching bags and 50 L tanks to represent small and larger scale production of *Artemia*.

		Cost	6 g of instar II (1 bag) A$/ day	36 g of instar II (6 bags) A$/ day	96 g of instar II (1 tank) A$/ day	192 g of instar II (2 tanks) A$/ day
Artemia cysts		$126.06/lb	0.34	2.05	5.44	10.88
Enrichment		$159/Kg	0.85	5.10	13.60	27.20
Electricity	Heat	$0.15/Kwh	5.40	5.40	5.40	5.40
	Light	$0.15/Kwh	2.93	2.93	2.93	2.93
Seawater	hatching	$0.10/L	1.80	10.8	15.00	30.00
	enriching	$0.10/L	1.20	7.20	10.00	20.00
Hypochlorite		$85/25L	0.34	2.04	5.44	10.88
Labour		$30/h	75.00	105.00	75.00	81.00
TOTAL		**A$**	**87.86**	**140.51**	**132.81**	**188.29**
Price/gram		A$	14.64	3.90	1.38	0.98

*Heater: 2.5Kw duty cycle at 5.2 h per day; Light: 24 40watt fluorescents lights at 24 h per day.

**Artemia* enriched at 100, 000 nauplii ml^{-1} therefore 1 g of *Artemia* enriched in 3 L.

PROJECTED COST OF PRODUCING 1,000 AND 10,000 SEAHORSES FED *ARTEMIA* AT DIFFERENT AGES

Table 6.4. The number of instar II *Artemia* required to feed 1,000 and 10,000 seahorses of different ages. Seahorses were fed 5% body weight day^{-1}. The equivalent amount of *Artemia* cysts and their cost is as follows:

Seahorse age (days)	Number of *Artemia*	g of cysts required to feed 1,000 seahorses / day	Cost per 1,000 A$/ day	Cost per 10,000 A$/ day
5	317567.6	1.095061	**16.03**	**15.11**
7	456081.1	1.572693	**23.02**	**21.70**
14	905405.4	3.122088	**45.70**	**43.08**
21	2033784	7.013048	**24.40**	**96.78**
35	3993243	13.7698	**47.92**	**134.94**
49	7736486	26.67754	**92.83**	**210.75**
56	8800676	30.34716	**105.61**	**239.74**
63	10023649	34.56431	**120.28**	**273.06**
91	10425676	35.95061	**125.11**	**284.00**
105	20743243	71.52842	**344.76**	**479.24**
119	22584459	77.87745	**375.36**	**521.78**
140	28513514	98.32246	**473.91**	**589.93**

* g of cyst required for 10,000 seahorses is 10x the number for 1,000 seahorses.

* costings used for 1,000 seahorses is based on hatching *Artemia* under small scale operations (6L hatching bags) and costings for 10,000 seahorses based on larger scale production (50 L tanks).

b) *COPEPODS*

ISSUES ASSOCIATED WITH THE USE OF COPEPODS

Benefits

- Can maintain population with a breeding stock;
- Do not require expensive enrichment diets.

Disadvantages

- Need backup tanks to maintain a breeding population;
- Grow in batch cultures;
- Low density cultures
- 2 to 3 week turnaround and 1 500 L tank only supplies feed for 2 - 3 days, therefore need a large number of tanks;
- Are cannabilistic;
- Time consuming process;
- Cultures prone to crashing;
- Algal culture facility to feed live algae to give optimal production.

COSTS INVOLVED WITH CULTURE

The costs of copepods include culture facilities, maintenance and running costs and feed costs.

Facility costs include: temperature controlled room, 500 L tanks for batch culture, fluorescent lighting, air lines, fresh and saltwater supply. Also includes algal culture facilities.

Maintenance costs include: tank fittings, pumps and hoses, airlines, light tubes. substrate screens for the copepods and screens and buckets for harvesting and running costs include: electricity, fresh and saltwater and labour.

FEED PREPARATION

Copepod culture

- Batch culture in 500 L tanks.

- At a density of 20 - 50 copepods/ml.
- Food concentration $5x10^4$ to $2x10^5$ cell ml^{-1} corresponding to water transparency of 7 - 10 cm.
- Generation time of 8 to 11 days.

Harvesting feed

- Substrate screens removed from tanks and copepods washed off through a series of screens to separate copepodids from adults, which were returned to the tanks.
- Copepodid number counted by subsamples under a microscope to ensure feed rates.

PROJECTED COST OF PRODUCING COPEPODS

For the purpose of this analysis the cost of the facility and maintenance costs have been excluded and the cost of purchasing algae is $80 / Kg.

Table 6.5. The projected cost of producing copepodids.

		Price	500 L tank ($2x10^6$ copepods) A$/day	2 x 500 L tank ($4x10^6$ copepods) A$/day
Electricity	Heat	$0.15/Kw	3.60	3.60
	Light	$0.15/Kw	0.0036	0.0036
seawater		$0.10/L	672	1344
labour		$30/h	75.00	90.00
Feed	algae	$80/ 1kg (dry)	1.92	3.84
TOTAL		**A$**	**750.60**	**1,437.604**
Price/5,000 copepod		A$	1.87	1.79

*Seawater: 500 L plus 1% water change per day plus 1 full water change a month. Heater: 2.5Kw duty cycle at 5.2 h per day; Light: 6 40watt fluorescent at 24 h per day. Feed: algae (8^4 cell ml^{-1} ~ 2 g dry weight).

*Copepods have a generation time of 10 days and 1 tank will only provide feed for 1 to 2 days therefore a further 12 tanks are needed for each culture.

PROJECTED COST OF PRODUCING 1,000 AND 10,000 SEAHORSES OF DIFFERENT AGES FED COPEPODS

Table 6.6. The number of copepods required to feed 1,000 and 10,000 seahorses 5% body weight day^{-1} and the feed cost.

Seahorse age (days)	Number of copepods / day	Cost per 1,000 A$/ day	Cost per 10,000 A$/ day
5	5465.116	**2.04**	**20.43**
7	7848.837	**2.93**	**29.35**
14	15581.4	**5.83**	**58.27**
21	35000	**13.09**	**130.9**
35	68720.93	**25.70**	**257.01**
49	133139.5	**49.79**	**497.94**
56	151453.5	**56.64**	**566.43**
63	172500	**64.51**	**645.15**

*Copepod number to feed 10,000 seahorses is 10x the number for 1,000 seahorses.

c) *BIOFOULING*

ISSUES ASSOCIATED WITH THE USE OF BIOFOULING

Benefits

- Do not require a culture facility or feed (eg algae or enrichments).

Disadvantages

- Collection time;
- Cost of net panels;
- Seasonally dependent;
- Requires an area with appropriate biofouling species;
- Requires marine lease infrastructure to securely support panels;
- Permits may be required.

COSTS INVOLVED WITH COLLECTION

The main costs of biofouling crustaceans will be associated with harvesting and include: collection buckets, net panels and labour (travel and collection).

Net panels are constructed from 15 mm electrical conduit ($1.30/m), 90° elbows ($0.85), and 20mm bar rope net ($12m^2) and the bottom of panels are filled with sand to keep them weighed down.

A small net panel (20 x 10 cm) costs $4.42 and a larger net panel (40 x 20 cm) costs $6.16.

FEED PREPARATION

After harvesting the net panels are placed directly into the seahorse tanks.

PROJECTED COST OF BIOFOULING CRUSTACEANS

For the purpose of this analysis the cost of storage and maintenance of collection buckets have been excluded.

Table 6.7. The projected costs of collecting biofouling crustaceans.

		Price	60 small net panels A$/day	120 small net panels A$/day	60 large net panels A$/day	120 large net panels A$/day
Electricity	heat	$0.15/Kwh	0.0	0.00	0.00	0.00
	light	$0.15/Kwh	0.00	0.00	0.00	0.00
seawater		$0.10/L	0.00	0.00	0.00	0.00
Net panel	small	$4.42	38.22	76.44	-	-
	big	$6.16	-	-	53.04	106.08
labour		$30/h	60.00	90.00	60.00	90.00
TOTAL		**A$**	**98.22**	**166.44**	**113.04**	**196.08**
Price/panel		$	1.63	1.38	1.88	1.63

*net panels degrade after 90 days and are replaced. The depreciated cost was used in calculations and are $0.049 and $0.068 for small and large panels respectively.

*Nets are allowed to recolonise for 2 weeks therefore for every panel used there is another 13.

*For this exercise nets were kept at Van Dieman Aquaculture therefore 1 hour of labour is travel.

PROJECTED COST OF PRODUCING 1,000 AND 10,000 SEAHORSES OF DIFFERENT AGES FED BIOFOULING

Table 6.8. The number of biofouling crustaceans required to feed 1,000 and 10,000 seahorses 5% body weight day^{-1} and the feed cost.

Seahorse age (days)	Size of prey consumed (mm)	Weight of feed on small panel(g)	Weight eaten by seahorses on small panel (g)	Number of small panels required/ day	Cost per 1,000 A$/ day	Number of large panels required/ day	Cost per 10,000 A$/ day
5	0.0 - 0.3	544.8	39.42372	14	**22.96**	7	**13.23**
21	0.0 - 0.59	2962	136.1747	22	**36.08**	11	**20.79**
49	0.0 - 0.79	7246.6	175.4647	41	**67.24**	21	**36.69**
91	0.2 - >1.1	27197	630.9771	43	**70.52**	22	**41.58**
147	0.3 - >1.1	32331	597.527	54	**88.56**	27	**51.03**
175	0.4 - >1.1	43459	499.559	86	**119.54**	44	**83.16**
203	0.3 - >1.1	66320	597.527	111	**154.29**	56	**105.84**

d) AMPHIPODS

ISSUES ASSOCIATED WITH THE USE OF AMPHIPODS

Benefits

- Do not require a culture facility or feed (eg algae or enrichment diets);
- Can be stored frozen.

Disadvantages

- Need a sorting area;
- Time required to collect, sort into sizes and package for freezing;
- Permits for collection;
- Seasonally dependent.

COSTS INVOLVED WITH COLLECTION

The main costs will be associated with harvesting and include labour (travel and collection) sorting out into sizes, packaging and freezing.

FEED PREPARATION

Amphipods are thawed and washed thoroughly with freshwater.

PROJECTED COST OF AMPHIPODS

For the purpose of this analysis the infrastructure and maintenance costs have been excluded.

Table 6.9. Projected cost of amphipod collection.

		Price	5 Kg (wet) A$/ day	15 Kg (wet) A$/ day
Electricity	Heat	$0.15/Kw	0.0	0.00
	Light	$0.15/Kw	0.00	0.00
seawater		$0.10/L	0.00	0.00
labour		$30/h	180.00	210.00
TOTAL		**$**	**180.00**	**210.00**
Cost/gram		$	0.036	0.014

*dry weight 0.3-0.4 mm amphipod, 0.59mg. Wet weight is 0.7375mg.

*There are 1,356 (0.3 - 0.4 mm) amphipods in a gram

PROJECTED COST OF PRODUCING 1,000 AND 10,000 SEAHORSES FOR DIFFERENT AGED SEAHORSES FED AMPHIPODS

Table 6.10. The number of frozen amphipods required to feed 1,000 and 10,000 seahorses 5% body weight day^{-1} and the feed cost.

Seahorse age (days)	Number of amphipods fed/ day	Cost per 1,000 A$/ day	Cost per 10,000 A$/ day
35	10016.95	**0.26**	**2.65**
49	19406.78	**0.51**	**5.15**
56	22076.27	**0.58**	**5.86**
63	25144.07	**0.66**	**6.67**
91	26152.54	**0.69**	**6.94**
105	52033.9	**1.38**	**13.81**
119	56652.54	**1.50**	**15.04**
140	71525.42	**1.89**	**18.98**
147	84559.32	**2.24**	**22.44**
175	102881.4	**2.73**	**27.31**
203	123881.4	**3.28**	**32.88**

* number of amphipods consumed by 10,000 seahorses is 10x that of 1,000 seahorses.

e) MYSIDS

COSTS INVOLVED WITH COLLECTION

Frozen mysids can be purchased frozen.

PROJECTED COST OF PRODUCING MYSIDS

Mysids are purchased at $20/Kg. The wet weight of mysids is 0.0375g therefore the price per gram (27 mysids) is $0.02.

PROJECTED COST OF PRODUCING 1,000 AND 10,000 OF DIFFERENT AGED SEAHORSES FED MYSIDS

Table 6.11. The number of frozen mysids required to feed 1,000 and 10,000 seahorses 5% body weight day^{-1} and the feed cost.

Seahorse age (days)	Number of mysids fed/ day	Cost per 1,000 A$/ day	Cost per 10,000 A$/ day
91	5143.333	**3.81**	**38.09**
105	10233.33	**7.58**	**75.80**
119	11141.67	**8.25**	**82.53**
140	14066.67	**10.42**	**104.19**
147	16630	**12.32**	**123.18**
175	20233.33	**14.98**	**149.87**
203	24363.33	**18.05**	**180.46**

*Number of mysids consumed by 10,000 seahorses is 10x that of 1,000 seahorses

6.2.2. SUMMARY OF PROJECTED COSTS

Table 6.12. The cost of feeding 1,000 and 10,000 different aged seahorses with *Artemia*, copepods, biofouling crustaceans, frozen amphipods and frozen mysids at a feed rate of 5% body weight.

day^{-1}.	*Artemia*		Copepods		Biofouling		Amphipods		Mysids	
Seahorse age (days)	per 1,000 (A$)	Per 10,000 (A$)	per 1,000 (A$)	Per 10,000 (A$)	per 1,000 (A$)	Per 10,000 (A$)	per 1,000 (A$)	Per 10,000 (A$)	per 1,000 (A$)	Per 10,000 (A$)
5	**16.03**	**15.11**	**2.04**	**20.43**	**22.96**	**13.23**				
7	**23.02**	**21.70**	**2.93**	**29.35**						
14	**45.70**	**43.08**	**5.83**	**58.27**						
21	**24.40**	**96.78**	**13.09**	**130.9**	**36.08**	**20.79**				
35	**47.92**	**134.94**	**25.70**	**257.01**			**0.26**	**2.65**		
49	**92.83**	**210.75**	**49.79**	**497.94**	**67.24**	**36.69**	**0.51**	**5.15**		
56	**105.61**	**239.74**	**56.64**	**566.43**			**0.58**	**5.86**		
63	**120.28**	**273.06**	**64.51**	**645.15**			**0.66**	**6.67**		
91	**125.11**	**284.00**			**70.52**	**41.58**	**0.69**	**6.94**	**3.809877**	**38.09**
105	**344.76**	**479.24**					**1.38**	**13.81**	**7.580247**	**75.80**
119	**375.36**	**521.78**					**1.50**	**15.04**	**8.253086**	**82.53**
140	**473.91**	**589.93**					**1.89**	**18.98**	**10.41975**	**104.197**
147					**88.56**	**51.03**	**2.24**	**22.44**	**12.31852**	**123.18**
175					**119.54**	**83.16**	**2.73**	**27.31**	**14.98765**	**149.87**
203					**154.29**	**105.84**	**3.28**	**32.88**	**18.04691**	**180.46**

6.2.3. PROJECTED COST OF PRODUCING 1,000 AND 10,000 SEAHORSES TO MARKET SIZE

Presently seahorses are cultured for a period of six months, at which stage they are available for sale to aquariums or other users. During this period the seahorses are fed enriched *Artemia* until 5 months of age and then weaned onto frozen mysids.

Based on these studies feasible feeding regimes can be developed utilising alternative feed sources to either totally or partially replace the reliance on *Artemia*. Different feeding regimes are required as some feeds are seasonally dependent i.e. live biofouling can only be fed during the period from October to March. Biofouling could however be collected during summer and fed out frozen over the winter months.

Suggested feeding regimes and the cost of feeding 1,000 and 10,000 seahorses to market size fed each of the feeding regimes are set out in the tables below:

Table 6.13. Possible feeding regimes for cultured seahorses.

	Weeks											
	2	4	6	8	10	12	14	16	18	20	22	24
Regime 1	Art	Art	Art	Art	Art	Art	Art	Art	Art	Art	Art	Art
Regime 2	Art	Art	Art	Art	Art	Art	My	My	My	My	My	My
Regime 3	Art	Art	Art	Art	Art	Art	Am	Am	Am	Am	Am	Am
Regime 4	Art	Co	Co	Co	Co	Co	Am	Am	Am	Am	Am	Am
Regime 5	Co	B	B	B	B	B	Am	Am	Am	Am	Am	Am

*Art - *Artemia*, Co - copepod, B – biofouling, Am - amphipod, My - mysid.

Table 6.14. The projected cost of producing 1,000 and 10,000 seahorses fed the different feeding regimes.

	For 1,000 seahorses (A$)	For 10,000 seahorses (A$)
Regime one	20,190.77	73,986.10
Regime two	10,830.84	43,141.02
Regime three	9,952.28	34,335.38
Regime four	3,488.75	33,807.23
Regime five	4,227.28	4,889.42

The prices above clearly demonstrate that the use of alternative food sources will bring about a significant reduction in the production costs of the pot-bellied seahorse.

Projected costs of producing seahorses to market size was determined using accumulated costs that were based on the costs detailed in this study (Figure 6.1 - 6.4).

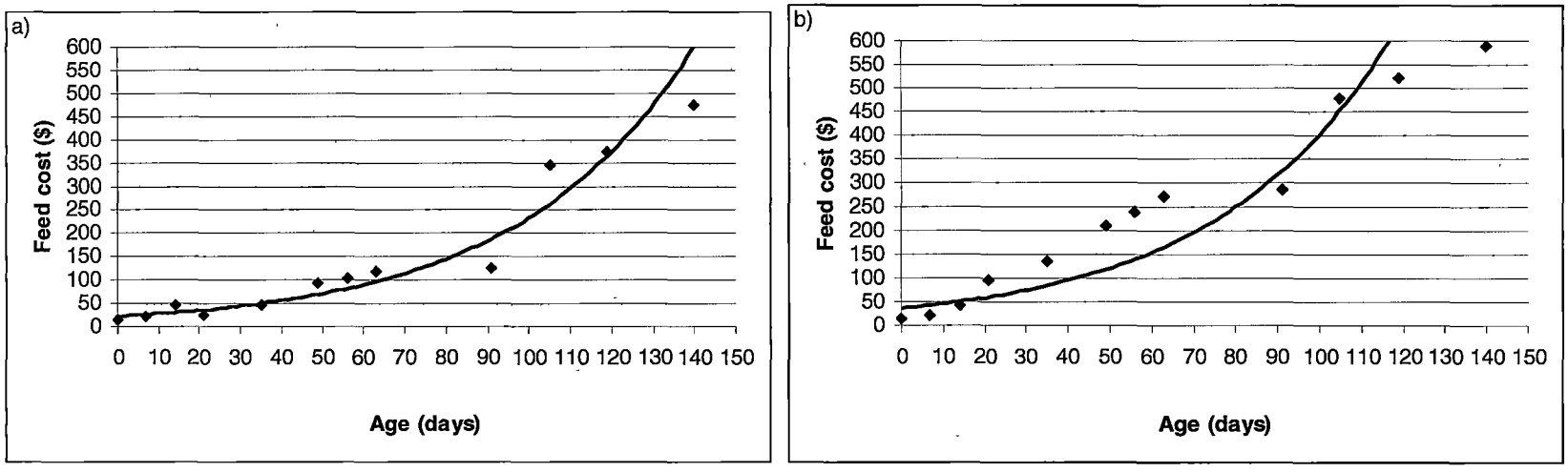


Figure 6.1. The daily cost of producing a) 1,000 and b) 10,000 seahorses fed enriched *Artemia*. An exponential line was fitted to the data and this was used to determine the accumulated costs for the missing data.

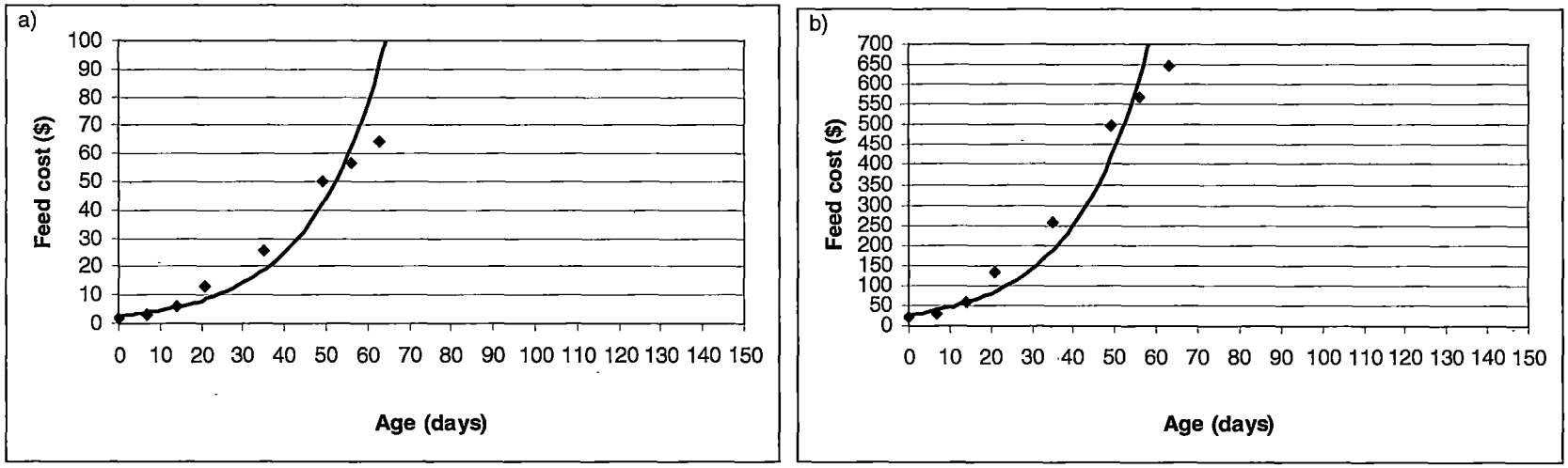


Figure 6.2. The daily cost of producing a) 1,000 and b) 10,000 seahorses fed copepods. An exponential line was fitted to the data and this was used to determine the accumulated costs for the missing data.

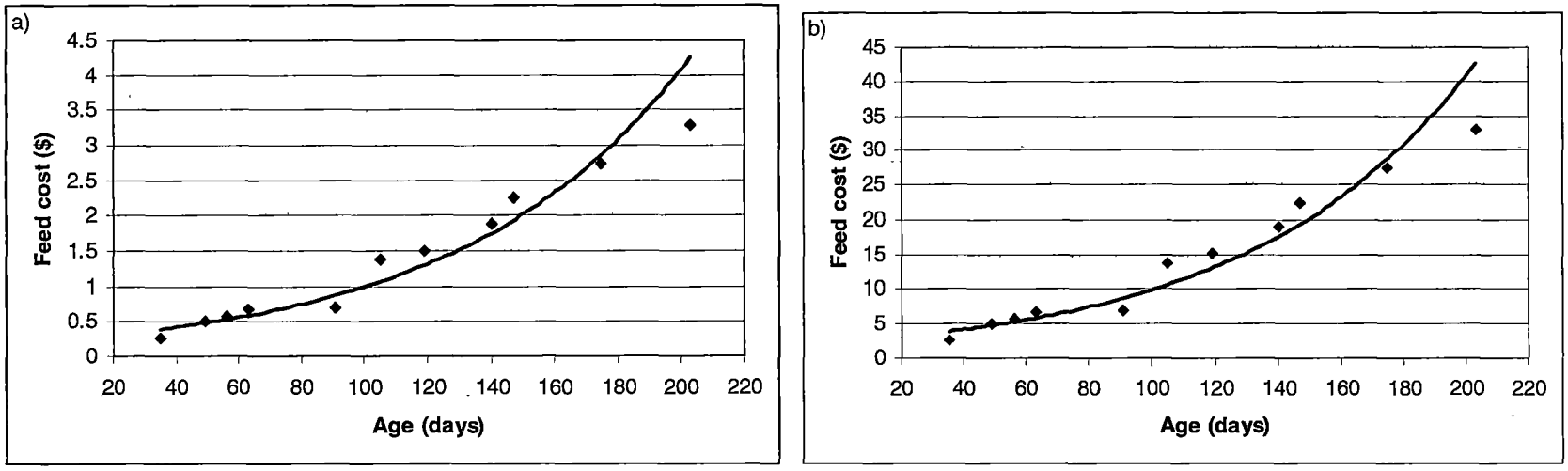


Figure 6.3. The daily cost of producing a) 1,000 and b) 10,000 seahorses fed amphipods. An exponential line was fitted to the data and this was used to determine the accumulated costs for the missing data.

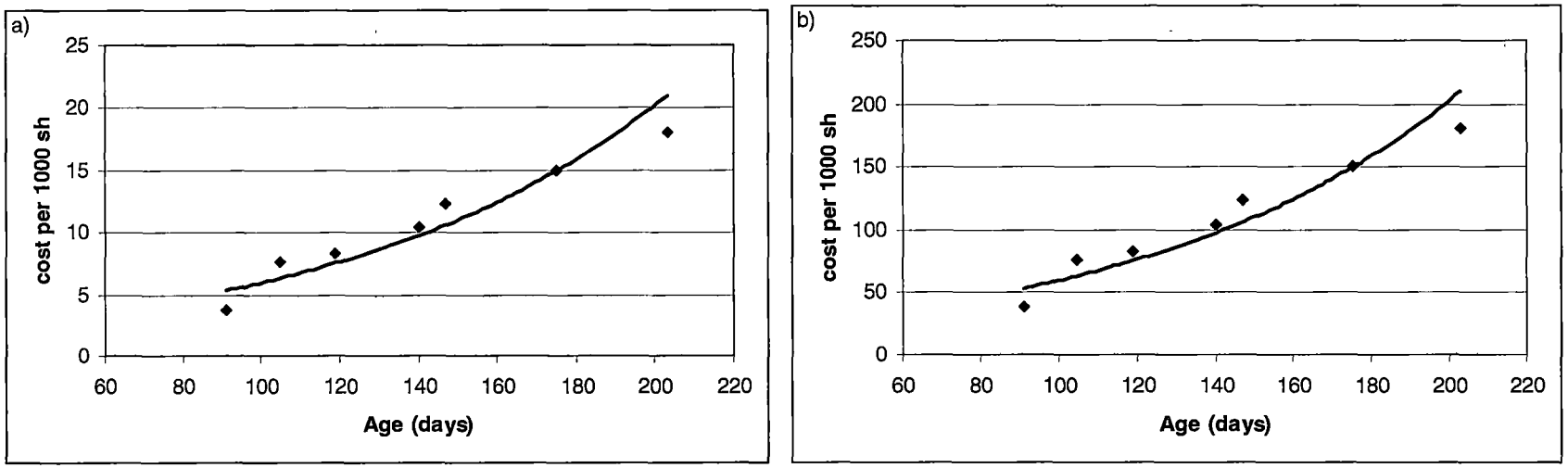


Figure 6.4. The daily cost of producing a) 1,000 and b) 10,000 seahorses fed mysids. An exponential line was fitted to the data and this was used to determine the accumulated costs for the missing data.

6.3. DISCUSSION

The above analysis of the cost of feeding 1,000 pot-bellied seahorses was carried out as part of the overall study of alternative feed diets. The analyses were based on research and information available as a result of these studies.

Certain assumptions have by necessity been made in arriving at the projected cost comparisons - eg. the costs of the facility/ies required for the culture, feed preparation and storage.

The cost of purchased *Artemia* cysts and enrichment protocols will include profit margins, whereas the cost of producing alternative feed sources does not.

One of the key costs associated with feed production and preparation is labour. If production of alternative feed sources was taken to a commercial level then there should be further economies of scale in respect thereto.

In summary, these studies show that an alternative feed/diet regime can be established with potentially significant reductions in the cost of the production of cultured pot-bellied seahorses.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1. General Discussion

The reliance on *Artemia* in the culture of pot-bellied seahorses can be reduced or at least partially replaced. In this study it was found that the growth and condition of 7 week old seahorses fed copepods was similar to those fed enriched *Artemia* and 13 week old seahorses weaned onto frozen mysids or amphipods over a 16 day period also showed growth similar to seahorses fed enriched *Artemia*. The growth of 17 week old seahorses fed biofouling crustaceans was similar to seahorses fed enriched *Artemia* but condition differed, with biofouling fed animals exhibiting significantly better condition. The studies also found that 3, 7 and 13 week old seahorses readily consumed biofouling crustaceans with the preferred prey (width) range of 3, 7, 13 and 21 week old seahorses being 0.17 - 0.51 mm, 0.18 - 0.73 mm, 0.18 - 1.27 mm and 0.18 - 1.35 mm respectively. The preferred prey type was copepods and amphipods for 3 and 7 week old seahorses and amphipods for 13 and 21 week old seahorses. In addition to promoting comparable growth and survival, alternative diets also appear less costly. A preliminary cost benefit analysis suggests such diets could be as low as 25% of the cost of producing *Artemia*.

Taking these findings into account, a possible feeding protocol for pot-bellied seahorses could be to feed newborn seahorses *Artemia* and from 3 weeks onwards replace *Artemia* with cultured copepods or biofouling crustaceans. At week 13, frozen diets could then be introduced over a 16 day weaning period.

This study showed that enrichment diets had no significant affect on the growth of seahorses, however, there may be longer term health implications and it is this possibility that makes enriching *Artemia* beneficial. The enrichment chosen as a reference diet in this study was Algamac 3050 due to its high protein and lipid content and popularity in other research trials. It was also noted during the gut content analysis study that newborn seahorses fed biofouling crustaceans for 5 days positively selected copepods in the size

range of 0.14 - 0.4 mm. It is possible that *Artemia* could be replaced or partially replaced from first feed onwards by an alternative diet of cultured copepods or biofouling crustaceans.

When developing a feeding protocol for the pot-bellied seahorse the size of prey and weaning time is important. Size composition of a new diet should be assessed to determine at what age seahorses can be fed a particular diet and this study suggests that seahorses should be weaned on to novel diets over a 16 day period, as they exhibit better growth when subjected to a slow changeover rather than a fast or abrupt changeover period. However, lower growth may be offset economically by abrupt weaning and this should be investigated further. Time to market size versus cost of production is an important consideration when deciding on an appropriate weaning protocol, and such comparisons deserve more attention within an operational context.

The relationship between seahorses and their preferred prey size is important in determining appropriate feeds for different aged seahorses. It was found that seahorses of different age groups eat a range of prey sizes and while large seahorses consume increasingly larger prey they also continue to consume small prey. Thus, while larger seahorses are not restricted to prey size younger seahorses are limited to smaller prey sizes. There was a strong relationship between prey width and total length of the seahorse and between prey width and gape height of the seahorse. The preferred attribute to determine a seahorses feed size is total length due to the fact that total length is an easier measure to record.

The use of alternative live feeds to replace *Artemia* in the culture of pot-bellied seahorses is not the preferred longtermoption as all live feeds are plagued with similar problems. Live feeds are possible sources of contaminants. Production in the wild is unreliable and harvesting of wild stock can be time consuming, dangerous and subject to seasonal conditions. In addition Tasmanian waters are not very productive. Government

regulations, which vary in different countries, restrict the species which can be cultured and (DPIWE) permits are required in Australian states to collect most species of crustaceans.

The culture of alternative live feeds would ensure availability, however production remains problematic. For example, the culture of copepods has been attempted a number of times but cultures tend to display low densities, slow generation times and are prone to crashes, as seen in this study.

Although biofouling crustaceans appear very promising in performance there are a few concerns to address. Some biofouling crustaceans are easy to remove from the collection panel (as seen when changing nets on salmon farms) while others are difficult to remove without causing damage to them. To ensure the well being of biofouling crustaceans net panels are placed directly into seahorse tanks and because biofouling can not be sorted there may be organisms present that could potentially harm pot-bellied seahorses; for example, stings from cnidarians and attacks by polycheate worms.

Despite these problems the use of biofouling crustaceans raises the potential to culture pot-bellied seahorses over the summer periods in sea cages and then transfer back into tanks for further ongrowing. While the production occurs year-round the majority of seahorses are bred over the late spring to summer period. Consequently early juveniles could commence life in tanks, be transferred to sea cages to feed on live biofouling and then transferred back into tanks to be weaned onto frozen diets and ongrown to market size. Cage culture is being trailled in a number of countries including New Zealand (Woods, pers comm.) and Singapore (Purser, pers comm.) with varying success.

The preferred strategy would be to wean seahorses onto frozen diets in order to reduce the reliance on *Artemia* and other live diets in the culture of pot-bellied seahorses. In the present study seahorses were successfully weaned

onto frozen amphipods and mysids at 13 weeks old. A number of studies have suggested that fish more readily accept novel diets when they are younger. If seahorse relationship to prey size is taken into account and a diet could be harvested and frozen, seahorses could potentially be weaned onto frozen diets at a younger age, thus reducing the length of time seahorses need to be fed on live diets. Possible frozen diets include amphipods and biofouling crustaceans, both of which can be found in abundance over the summer months (October to April), easily sorted into appropriate size categories and frozen and still retain their key characteristics on thawing. In the case of biofouling, it is difficult to remove some species from the net panels alive but as they are to be frozen they could simply be washed off.

At present frozen mysids are widely used in seahorse culture, however there is concern about the sustainability of mysid fisheries (www.dpiwe.tas.gov.au). A permit issued under section 14 of the Living Marine Resources Management Act 1995 is required to collect mysids in Tasmania. At present the permits are restricted to the University of Tasmania, Seahorse World Pty. Ltd. and Seahorse Australia Pty. Ltd. under a system, which is reviewed every 5 years and mysid population numbers are being assessed. An alternative to mysids is amphipods. In this study amphipods promoted good growth and condition in seahorses, however there are many different types and their nutritional profiles need to be further assessed before preferred species for seahorses can be determined and legislation for collection put into place.

The ultimate aim to reduce the reliance of seahorses on *Artemia* is to formulate an artificial diet. To formulate an artificial diet a number of requirements need to be met:

- the size of the diet;
- determine what attracts a seahorse to a particular diet;
- determine at what age seahorses are capable of digesting certain compounds;

- and the time required to wean seahorses onto a new diet.

The time it takes a seahorse to accept a new diet, the preferred prey size and prey types of different aged seahorses have been determined in this study and, from the work undertaken on the development of the digestive tract and ontogenetic development of digestive enzymes, it could be suggested that seahorses have the capability of digesting an artificial diet from newborn onwards. It has been demonstrated that seahorses are released with a developed digestive tract made up of a buccal cavity, an oesophagus with what seems to be gastric cells in the latter half, an intestine with epithelial lining, rectum and an already formed pancreas and liver. It was also found that digestive enzymes are produced from the pancreas and from cell clusters within the intestinal epithelium and digestive enzymes were present before first feed, which indicates that seahorses are already capable of protein, lipid and carbohydrate digestion.

Although the digestive tract of the seahorse is equipped to assimilate exogenous sources of food from the day of release from the male's pouch, activity of the enzymes were shown to change with age as amylase activity continued to increase until day 28 and lipase and trypsin activity continued to increase until day 35. The intestinal valve, which separates the intestine from the rectum only becomes apparent on day 7, the intestine starts to loop around itself on day 21 and the mean gut epithelia becomes thicker with age increasing the surface area available for enzyme secretion and hydrolysis of nutrients.

Theoretically, based on the evidence that enzymes are present from release onwards, newborn seahorses could utilize artificial diets. However, when histological development and ontogenetic development of digestive enzymes are looked at together it was found that enzyme activity tended to peak around the same time the gut started to loop. This timing also coincided with 'settlement' as seahorses do not tend to start sitting on the bottom of the tanks until they are 3 to 5 weeks old. It may be that seahorses are not fully

developed and capable of accepting and utilizing an artificial diet until they have changed from a pelagic to a more benthic existence. It is also important to determine the optimum daily feed rate to ensure good feed management. It was found that a ration in the range of 5% to 7.5% body weight day^{-1} promoted good growth however using a daily ration near 5% body weight day^{-1} would be more feed efficient.

In summary,

- there is a high demand for seahorses for the traditional medicine and aquarium markets;
- culture of pot-bellied seahorses is reliant on *Artemia* which are an expensive food source and, as a consequence, inhibit the production of seahorses;
- if pot-bellied seahorse culture is to be economically feasible alternative diets are required to totally or at least partially replace *Artemia*;
- the reliance of pot-bellied seahorses on *Artemia* can be totally or at least partially reduced;
- pot-bellied seahorses will accept alternative live diets with 3 to 9 week old seahorses consuming cultured copepods and 3, 7, 13, 21, 25 and 29 week old seahorses consuming harvested biofouling crustaceans;
- pot-bellied seahorses at an age of 13 weeks old can be weaned onto frozen diets and a weaning period of 16 days gives the best growth;
- possible frozen diets for pot-bellied seahorses are amphipods, mysids, biofouling crustaceans and copepods;
- seahorses remain stomachless throughout life;
- seahorses are released from the male's pouch with a developed digestive tract comprised of a buccal cavity, oesophagus, intestine and rectum and the liver and pancreas are already formed;

- other than the intestinal valve being developed by day 7, the intestine beginning to loop around itself on day 21 and the intestinal mucosa becoming denser with age there are no major developmental changes;
- there are what seem to be gastric cells in the lower half of the oesophagus and gut enzymes are produced within the pancreas and intestinal mucosa;
- seahorses are released from the male's pouch with the ability to digest lipids, proteins and carbohydrates;
- lipase, trypsin and amylase enzyme activity increases from before first feed onwards and tends to peak around 28 to 35 days after release;
- diet does not affect enzyme activity while ration level does, with enzyme activity increasing with an increasing ration.

The above studies demonstrate that *Artemia* can be replaced or supplemented in the culture of pot-bellied seahorses with alternative live feeds and that seahorses can be weaned onto frozen diets at an early age. When the potential cost and benefits of alternative diets were compared with *Artemia* it was found that there would be substantial cost savings by using alternative diets. The work on the preferred prey size and prey type of seahorses, weaning times, development of the digestive tract and ontogenetic development of digestive enzymes has given a better understanding of the feeding behaviour and development of the pot-bellied seahorse and provided important information needed to formulate an artificial diet.

It is now essential that further studies are conducted to ensure the ongoing culture of pot-bellied seahorses is economically viable:

- develop feeding regimes for seahorses;
- ascertain characteristics of preferred prey items;
- assess the potential to culture alternative live feeds;
- assess the potential of growing seahorses in sea cages;

- determine the earliest age that seahorses can be weaned onto frozen diets;
- further work on histochemistry of digestive glands and mucosubstances;
- further studies on other digestive enzymes of seahorses;
- formulate and trial artificial diets on the growth and condition of seahorses.

7.2 . SUMMARY OF MAIN FINDINGS

7.2.1 MORPHOLOGICAL DESCRIPTION

Table 7.1. The weight, length, head length, snout length, gape height and eye diameter of different aged pot-bellied seahorses.

Age (Days)	Morphological measurements					
	Weight (g)	Length (mm)	Head Length (mm)	Snout Length (mm)	Gape Height (mm)	Eye diameter (mm)
0	0.008	14				
5	0.009	16	3.71	1.82	1.24	0.97
7	0.014					
14	0.027					
21	0.062	25	6.31	3.01	1.68	1.43
35	0.118					
49	0.229	36.37	9.35	4.72	2.15	2.08
56	0.265					
63	0.297					
91	0.308	50	11.82	5.54	2.77	2.72
105	0.614	74				
119	0.668					
140	0.844					
147	0.998					
175	1.214	80	14.29	5.71	3.91	3.46
203	1.462	87	15.48	6.52	4.73	3.26

7.2.2. FEED TYPE CONSUMED AND PREFERRED PREY SIZE.

Table 7.2. The feed types consumed and the preferred prey size range of different aged pot-bellied seahorses

Age (Days)	Potential diet sources	Preferred prey size (mm)
0	*Artemia*	
5	*Artemia* Biofouling crustaceans (copepods, amphipods)	0.14 - 0.4
7	Artemia	
14	*Artemia*	
21	*Artemia* Copepods Biofouling crustaceans (copepods, amphipods)	0.17 - 0.51
35	*Artemia* copepods	
49	*Artemia* Copepods Biofouling crustaceans (copepods, amphipods)	0.2 - 0.73
56	*Artemia* Copepods	
63	*Artemia* Copepods	
91	*Artemia* Biofouling crustaceans (amphipods, caprellids) Frozen amphipods and mysids	0.18 - 1.27
105	*Artemia* Frozen amphipods and mysids	
119	*Artemia* Frozen amphipods and mysids	
140	*Artemia* Frozen amphipods and mysids	
147	*Artemia* Biofouling crustaceans (amphipods and caprellids) Frozen amphipods and mysids	0.18 - 1.35
175	*Artemia* Biofouling crustaceans (amphipods and caprellids) Frozen amphipods and mysids	0.33 - 1.48
203	*Artemia* Biofouling crustaceans (amphipods and caprellids) Frozen amphipods and mysids	0.33 - 1.70

7.2.3. DIGESTIVE ENZYMES

Table 7.3. Summary of ontogenetic development of digestive enzymes of the pot-bellied seahorse.

Age (Day)	Enzymes			
	Amylase (mg protein^{-1} .seahorse^{-1})	Lipase (mg protein^{-1} .seahorse^{-1})	Trypsin (mg protein^{-1} .seahorse^{-1})	produced
0	0.089 ± 0.013	0.257 ± 0.023	0.137 ± 0.011	Gastric cells Pancreas Liver
0	0.171 ± 0.001	0.397 ± 0.066	0.211 ± 0.042	
3	0.189 ± 0.01	0.582 ± 0.032	0.284 ± 0.022	Gastric cells Pancreas Liver
7	0.137 ± 0.01	0.597 ± 0.061	0.159 ± 0.06	Gastric cells Pancreas Liver
14	0.207 ± 0.004	0.629 ± 0.112	0.536 ± 0.084	Gastric cells Pancreas Liver
21	0.227 ± 0.001	0.711 ± 0.004	0.77 ± 0.028	Gastric cells Pancreas Liver
28	0.26 ± 0.004	0.726 ± 0.053	0.901 ± 0.045	Gastric cells Pancreas Liver
35	0.253 ± 0.011	0.933 ± 0.069	1.267 ± 0.03	
42	0.248 ± 0.004	0.887 ± 0.049	1.19 ± 0.031	
49	0.252 ± 0.012	1.005 ± 0.089	1.065 ± 0.05	
56	0.222 ± 0.005	0.97 ± 0.05	0.848 ± 0.081	

7.2.4. HISTOLOGICAL DEVELOPMENT OF THE DIGESTIVE TRACT

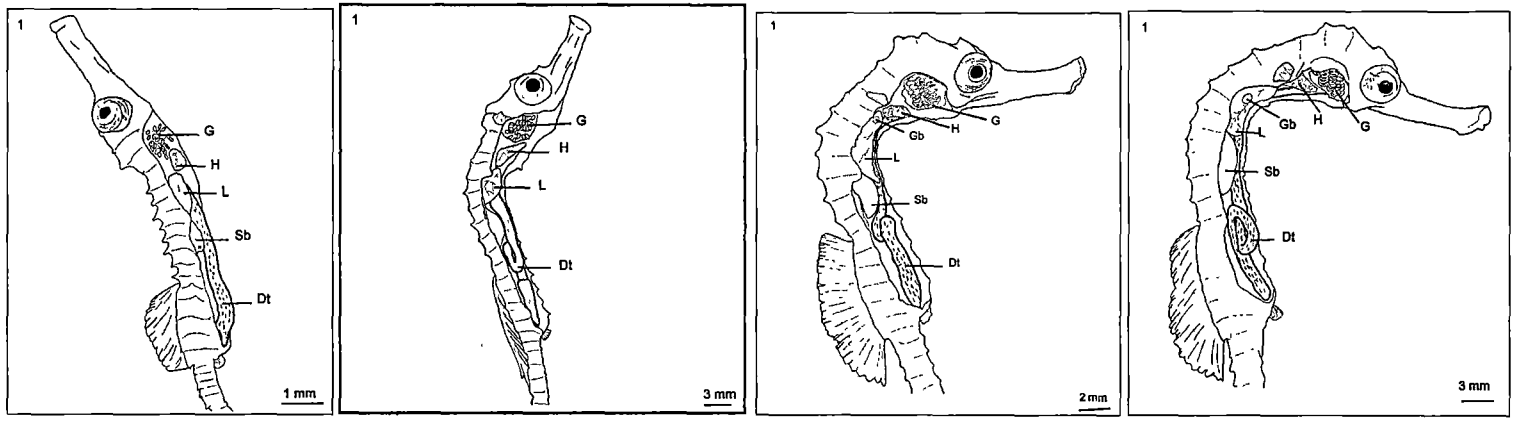


Figure 7.1. The histological development of the digestive tract of pot-bellied seahorses.

CHAPTER EIGHT

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CHAPTER NINE

APPENDIX

APPENDICIES

9.1. Determining the number of *Artemia* to hatch.

9.2.*Artemia* enrichment procedure. The dose rate and enrichment time.

9.3.Nutritional profile of pot-bellied seahorses and instar II *Artemia* nauplii.

9.4. Nutritional profiles of the algae, Algamac-3050, Protein Selco, Super Selco and Artemac Enrichment diets used in the *Artemia* Enrichment trial.

9.5. Initial and final mean (± SE) weight of seahorses in the *Artemia* enrichment trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

9.6. Initial and final mean (±S.E) weight of seahorses in each treatment in the ration trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

9.7. Determining the daily number of frozen amphipods and mysids that are needed in the weaning trial.

9.8. Initial and final mean (±S.E) weight of seahorses in the biofouling and *Artemia* dietary trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

9.9. Initial and final mean (±S.E) weight of seahorses in the copepod and *Artemia* dietary trial. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

9.10. Mean (±S.E) weight of seahorses at the beginning and end of the frozen diet trial. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

9.11. Total protein content

9.12. Enzyme calculations

9.13. Ages of seahorses in the feed trials

9.1. DETERMINING THE NUMBER OF *ARTEMIA* TO HATCH

- The total number of instar II *Artemia* in a gram is 245279.2 (Hatch rate of 80%) and the individual dry weight of an instar II is 0.00148 mg (Wardley, 2001).
- Average weight of seahorses used (start of trial) = 0.14 g
- Seahorses are to be fed 5 % body weight day^{-1} = 0.007g

A 0.14 g seahorse eats 7 mg of instar II *Artemia* at 0.00148 mg (dry weight)

$\therefore$ 7 mg / 0.00148 mg

= 4730 instar II *Artemia* are required for each seahorse

As there are 20 seahorses per tank and 18 tanks in total, 1702800 instar II *Artemia* are required per day and this equates to 6.9 g (1702800 / 245279.2) of cysts.

9.2. ENRICHMENT PROCEDURE. THE DOSE RATE AND ENRICHMENT TIME

- Transfer *Artemia* nauplii to an enrichment tank
- Density should not exceed 100,000 nauplii L^{-1} of water
- Aerate vigorously

Algamac 3050

- Blend Algamac for 5 minuties
- Add Algamac 3050 at a rate of 0.2 g L^{-1} (per 100 000 nauplii)
- Allow to enrich for 12 hours and feed out
- Re-enrich at a rate of 0.2 g L^{-1}

Super Selco

- Blend Super Selco for five minutes
- Add Super Selco at a rate of 600 mg/L
- Allow to enrich for 12 hours and feed out
- Re-enrich afternoon fed at a rate of 600 mg/L

Protein Selco

- Blend Protein Selco for five minutes
- Add Selco at a rate of 600 mg/L
- Allow to enrich for 12 hours and feed out
- Re-enrich afternoon fed at a rate of 600 mg/L

Artemac

- Blend Artemac for five minutes
- Add Artemac at a rate of 0.4 g/L
- Allow to enrich for 12 hours and feed out

Re-enrich afternoon fed at a rate of 0.2 g/L

9.3. NUTRITIONAL PROFILE OF POT-BELLIED SEAHORSES AND INSTAR II *ARTEMIA* NAUPLII

	Artemia	seahorses
GROSS CHEMICAL COMPOSITION	% dry weight	% dry weight
Protein	41-47 (1,3)	
Carbohydrate	11 (1,3)	
Lipid	21-23 (1,3)	
Ash	10 (1,3)	
ESSENTIAL COMPONENTS		
Amino acids	g/100g total a. acid	g/100g total a. acid
alanine	4.1 (3)	
arginine	8.2 (3)	
aspartate	9.5 (3)	
cystine		
glutamate	11.4 (3)	
glycine	5.1 (3)	
histidine	2.3 (3)	
isoleucine	5.7 (3)	
leucine	8.4 (3)	
lysine	7.8 (3)	
methionine	3.1 (3)	
phenylalanine	7.2 (3)	
proline	5 (3)	
serine	4.5 (3)	
threonine	4 (3)	
tryptophan		
tyrosine	5.6 (3)	
valine	4.4 (3)	
Lipids		
Fatty acid components	g/100g	% dry weight
C16 polyunsaturates	12.3 (2)	
18:2w6	64 (2)	9.3 (FRDC)
18:3w6		
18:3w3	44.5 (2)	0.0 (FRDC)
18:4w3		
18:5w3		
20:3w6 (ETA)		0.1 (FRDC)
20:4w6	tr (2)	
20:4w3 (ETA)		0.4 (FRDC)
20:5w3 (EPA)	2.4 (2)	6.3 (FRDC)
22:5w6 (DPA)	n.d.(2)	3.4 (FRDC)
22:6w3 (DHA)	n.d. (2)	12.2 (FRDC)
DHA/EPA ratio		

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9.4. NUTRITIONAL PROFILES OF THE ALGAE, ALGAMAC-3050, PROTEIN SELCO, SUPER SELCO AND ARTEMAC ENRICHMENT DIETS USED IN THE *ARTEMIA* ENRICHMENT TRIAL

	Isochrysis galbana	*Chaetoceros calcitrans*	*Tetrasemis suecica*	Algamac 3050	Protein selco	Super Selco	Artemac
GROSS CHEMICAL COMPOSITION	% dry weight	% dry weight	% dry weight	% dry weight	% dry weight	% dry weight	% dry weight
Protein	41% (1,9)	33% (2)	39% (1)	17.6 (11)	30 (12)		57 (13
Carbohydrate	5% (1,9)	17% (2)	8% (1)	15.9 (11)			12 (13)
Lipid	21% (1,9)	10% (2)	7% (1)	56.2 (11)	25 (12)	65 (12)	19 (13)
Mineral	13% (1)	29% (2)	23% (1)				
Ash				8.2 (11)	15 (12)	3 (12)	5 (13)
Moisture				2.1 (11)	5 (12)	65 (12)	7 (13)
calories (C/100g)				640.0 (11)			
Total	80% (1)	89% (2)	77% (1)				
ESSENTIAL COMPONENTS							
Amino acids	g/100g total a. acid	g/100g total a. acid	g/100g total a. acid	mg/100g			
alanine	9.7 (3)	7.7 (4)	8.7 (5)	750 (11)			
arginine	5.7 (3,9)	6.4 (4)	6.4 (5)	1650 (11)			
aspartate	9.9 (3)	11.4 (4)	10.0 (5)	1260 (11)			
cystine	0.5 (3)	n.d.	0.4 (5)				
glutamate	8.4 (3)	15.0 (4)	13.5 (5)	4180 (11)			
glycine	6.3 (3)	5.5 (4)	7.4 (5)	640 (11)			
histidine	1.9 (3,9)	2.3 (4)	2.2 (5)	240 (11)			
isoleucine	3.3 (3)	5.5 (4)	4.8 (5)	400 (11)			
leucine	10.2 (3,9)	9.1 (4)	3.2 (5)	700 (11)			
lysine	7.3 (3,9)	7.3 (4)	6.6 (5)	530 (11)			
methionine	3.2 (3,9)	2.3 (4)	2.0 (5)				
phenylalanine	4.4 (3,9)	5.9 (4)	5.9 (5)	420 (11)			
proline	6.7 (3,9)	n.d.	3.7 (5)	400 (11)			
serine	6.0 (3)	5.9 (4)	4.3 (5)	460 (11)			

	Isochrysis galbana	*Chaetoceros calcitrans*	*Tetrasemis suecica*	Algamac 3050	Protein selco	Super Selco	Artemac
threonine	5.0 (3)	5.9 (4)	3.6 (5)	440 (11)			
tryptophan	0.4 (3,9)	n.d.	0.5 (5)				
tyrosine	2.1 (3,9)	4.5 (4)	9.7 (5)	300 (11)			
valine	6.8 (3,9)	5.9 (4)	7.1 (5)	610 (11)			
Carbohydrates	g/100g	g/100g	g/100g				
rhamnose	1.0 (1)	1.1 (1)	0.3 (1)				
fucose	0.8 (1)	5.3 (1)	0.3 (1)				
ribose	48.2 (1)	10.9 (1)	3.8 (1)				
zylose	6.9 (1)	1.1 (1)	0.2 (1)				
arabinose	3.4 (1)	n.d.	0.7 (1)				
mannose	11.8 (1)	1.4 (1)	19.0 (1)				
galactose	13.7 (1)	12.4 (1)	32.2 (1)				
glucose	12.7 (1)	66.3 (1)	43.5 (1)				
inositol	1.5 (1)	1.6 (1)					
glycerol							
fructose ribitol/xylitol							
Lipids							
FATTY ACID COMPONENT	g/100g	g/100g	g/100g	%w/w			mg/g
saturates	37.0 (6)	30.2 (7)	26.8 (7)				
monosaturates	30.4 (6)	33.8 (7)	20.5 (7)				
C14 Myristate				8.85 (11)			
C16 polyunsaturates	0.4 (6)	13.4 (7)	17.2 (7)	26.6 (11)			
18:2w6	2.3 (6)	0.8 (7,10)	13.9 (7,10)				
18:3w6	0.2 (6)	0.4 (7,10)	2.7 (7,10)				
18:3w3	0.4 (6)	tr (7,10)	4.6 (7,10)				
18:4w3	8.0 (6)	0.5 (7,10)	4.8 (7,10)				
18:5w3	n.d. (6)	n.d. (10)	n.d.				
20:3w6 (ETA)				0.22 (11)			
20:4w6	0.1 (6,9)	5.7 (7)	2.1 (7)				

	Isochrysis galbana	*Chaetoceros calcitrans*	*Tetrasemis suecica*	Algamac 3050	Protein selco	Super Selco	Artemac
20:4w3	n.d.	0.2 (7,10)	0.1 (7,10)				
20:5w3 (EPA)	7.2 (6,9)	11.1 (7,10)	5.3 (7,10)	2.88 (11)			11.2 (13)
22:5w6 (DPA)	n.d.	n.d.	n.d.	17.04 (11)			
22:6w3 (DHA)	4.3 (6,9)	0.8 (7)	tr (7)	43.17 (11)			12.7 (13)
Sum of HUFA (mg/g dwt)					70 (12)	400 (12)	
DHA/EPA ratio					1 (12)	1 (12)	
PHOSPHOLIPIDS							
STEROLS				mg/100g			
beta-Sitosterol				19.7 (11)			
campesterol				10.6 (11)			
cholesterol				178 (11)			
stigmasterol				154 (11)			
Minerals	mg/dry weight	mg/dry weight	mg/dry weight				
Ca	16.2 (8)		20.8 (8)				
P	10.2 (8)		6.5 (8)				
Na	7.2 (8)		10.4 (8)				
K	5.6 (8)		12.0 (8)				
Cl	50.8 (8)		37.2 (8)				
Fe	3.6 (8)		10. (8)				
Mg	11.5 (8)		7.8 (8)				
Zn	0.6 (8)		1.5 (8)				
Mn	0.04 (8)		0.05 (8)				
Co	0.01 (8)		.0005 (8)				
Cu	0.2 (8)		0.6 (8)				
Vitamins							
cyanocobalamin (vitamin B12)				65.8 ug (11)			
pyridoxine (vitamin B6)				3.62 mg (11)			
riboflavine (vitamin B2)				1.65 mg (11)			
thiamin (vitamin B1)				2.4 mg (11)			
pteroylmonoglutamic acid							

	Isochrysis galbana	*Chaetoceros calcitrans*	*Tetrasemis suecica*	Algamac 3050	Protein selco	Super Selco	Artemac
biotin				237.00 ug (11)			
nicotinic acid							
pantothenic acid				10.1 mg (11)			
ascorbic acid (vitamin C)				71.3 mg (11)	20 mg (12)	8 mg (12)	
tocopherol (vitamin E)				<0.5 IU (11)	<1 mg (12)	<0.5 mg (12)	
B-carotene (provitamin A)				<100 IU (11)	150 IU (12)	150 IU (12)	
Choline				188 ug (11)			
Folic acid (/100g)				357.00 ug (11)			
inositol				180 (11)			
niacin				7.16 mg (11)			

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9.5. WEIGHT OF SEAHORSES IN THE *ARTEMIA* ENRICHMENT TRIAL

Initial and final mean (± SE) weight of seahorses in the *Artemia* enrichment trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

Treatment	Weight (g) Start	Weight (g) End
Unenriched	0.246 ± 0.007^{a}	0.600 ± 0.028^{b}
Algae	0.244 ± 0.006^{a}	0.578 ± 0.024^{b}
Super Selco	0.233 ± 0.007^{a}	0.512 ± 0.024^{b}
Protein Selco	0.245 ± 0.008^{a}	0.574 ± 0.023^{b}
Artemac	0.242 ± 0.007^{a}	0.636 ± 0.027^{b}
Algamac 3050	0.229 ± 0.005^{a}	0.614 ± 0.025^{b}

9.6. WEIGHT OF SEAHORSES IN THE RATION TRIAL.

Initial and final mean (±S.E) weight of seahorses in the ration trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

Ration	Weight (g) Start	Weight (g) End
25%	0.0668 ± 0.0022	0.205 ± 0.0078
50%	0.0705 ± 0.0024	0.2494 ± 0.0077
75%	0.0690 ± 0.0022	0.2589 ± 0.0083
100%	0.0718 ± 0.0024	0.3341 ± 0.0116
125%	0.0696 ± 0.0023	0.3914 ± 0.0175
150%	0.0721 ± 0.0023	0.4143 ± 0.0147

9.7. DETERMING THE DAILY NUMBER OF FROZEN AMPHIPODS AND MYSIDS THAT ARE NEEDED IN THE WEANING TRIAL

- If the weight of seahorses = 0.3 g
- and seahorses were fed 5 % body weight day^{-1} = 0.015g day^{-1}

Then:

The number of amphipods needed per day is:

A 0.3 g seahorse eats 0.015 g of amphipods at 0.00059 g (dry weight)

$\therefore$ 0.015 g / 0.00059 g

= 25.42 amphipods are required for each seahorse

As there are 15 seahorses per tank 381 amphipods are needed each day.

The number of mysids needed per day is:

A 0.3 g seahorse eats 0.015 g of mysids at 0.003 g (dry weight)

$\therefore$ 0.015 g / 0.003 g

= 5 amphipods are required for each seahorse

As there are 15 seahorses per tank 75 mysids are needed each day.

9.8. WEIGHT OF SEAHORSES IN THE BIOFOULING AND *ARTEMIA* DIETARY TRIAL.

Initial and final mean (±S.E) weight of seahorses in the biofouling and *Artemia* dietary trial. Means which did not significantly differ in the Tukey's HSD test share the same common superscript.

Treatment	Weight (g) Start	Weight (g) End
Artemia	0.6525 ± 0.0193	1.3068 ± 0.0442
Biofouling	0.6685 ± 0.01767	1.4618 ± 0.0638

9.9. WEIGHT OF SEAHORSES IN THE COPEPOD DIET TRIAL.

Initial and final mean (±S.E) weight of seahorses in the copepod and *Artemia* dietary trial. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

Time	Treatment	
	Artemia Mean (±S.E)	Copepod Mean (±S.E)
Start	$0.102 \pm .00202^{a}$	0.101 ± 0.00226^{a}
Week 2	0.1301 ± 0.00331^{b}	0.1106 ± 0.00341^{b}
Week 4	0.2376 ± 0.00789^{c}	0.2161 ± 0.00729^{c}
Week 6	0.2967 ± 0.00953^{d}	0.2905 ± 0.01030^{d}

9.10. WEIGHT OF SEAHORSES IN THE FROZEN AMPHIPOD AND KRILL WEANING TRIALS.

Table 9.10a. Initial mean (±S.E) weight of seahorses in the frozen diet trials. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

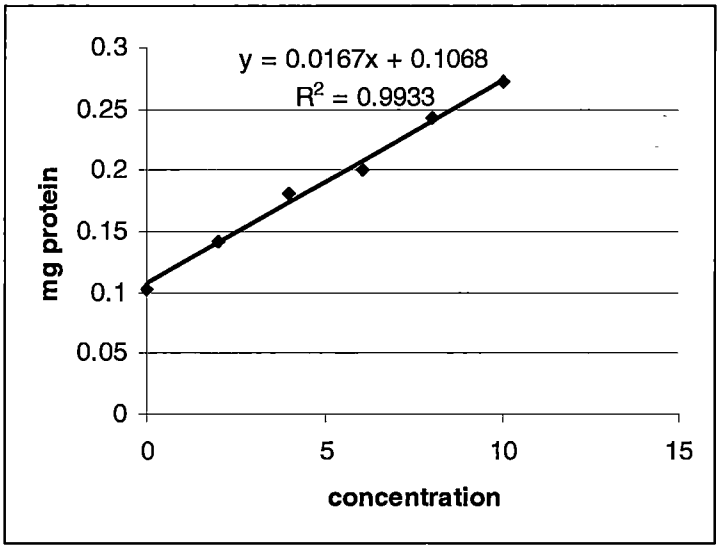
	Artemia only	10 day weaning period	16 day weaning period	No weaning period
Amphipod				
Start weight	0.3026 ± 0.007^{a}	0.2888 ± 0.008^{a}	0.3123 ± 0.01^{a}	0.3086 ± 0.0104^{a}
Mysid				
Start weight	0.3046 ± 0.009^{a}	0.2913 ± 0.008^{a}	0.2908 ± 0.008^{a}	0.302 ± 0.009^{a}

Table 9.10b. Final mean (±S.E) weight of seahorses in the frozen diet trials. Means which did not significantly differ in Tukey's HSD test share the same common superscript.

	Artemia only	10 day weaning period	16 day weaning period	No weaning period
Amphipod				
End weight	0.9026 ± 0.009^{a}	0.8117 ± 0.009^{c}	0.9978 ± 0.011^{a}	0.7884 ± 0.011^{c}
Mysid				
End weight	$0.9046 \pm .0102^{b}$	0.8171 ± 0.0093^{b}	0.9637 ± 0.0102^{b}	0.8038 ± 0.0069^{b}

9.11. TOTAL PROTEIN CONTENT

The total protein concentration of each enzyme extract was measured in accordance to Bradford (1976), with bovine serum albumin (BSA) as the standard and with Bradford's dye. Enzyme activity was measured at an absorbance of 595nm.



9.12. ENZYME CALCULATIONS

Readings from the spectrophotometer were converted to AU / min by:

$\Delta abs / \Delta$ time = €CL

Where: € = extinction coefficient $m^{-1}cm^{-1}$

L = light path - 0.62 cm for a microplate

AMYLASE CALCULATION

Au/min (e.g 0.006) = € (10130 $m^{-1}cm^{-1}$) x C x L (0.62 cm)

Therefore C = (Au/min) / € x L (0.62

C = 0.006 / 10130 $m^{-1}cm^{-1}$ x 0.62cm

C = 0.006 / 6280.6

= 9.55 x 10^{-7} M/L

= 0.955 µmol/ L /min (convert to µmol x 1000 000)

= 0.0096 µmol / ml / min (convert to ml x 0.001)

5 µl in assay

therefore 0.0096 µmol / min / 5 µl

to convert to ml x 200

0.191 µmol / min / ml (TOTAL ACTIVITY)

TRYPSIN CALCULATION

Au/min (e.g 0.006) = € (9300 $m^{-1}cm^{-1}$) x C x L (0.62 cm)

C = 0.006 / 9300 $m^{-1}cm^{-1}$ x 0.62cm

C = 0.006 / 5766

= 1.04 x 10^{-6} M/L

= 1.04 µmol/ L /min (convert to µmol x 1000 000)

= 0.00104 µmol / ml / min (convert to ml x 0.001)

10 µl in assay

therefore 0.00104 µmol / min / 10 µl

to convert to ml x 100

0.104 µmol / min / ml (TOTAL ACTIVITY)

LIPASE CALCULATION

Au/min (e.g 0.006) = € (9200 $m^{-1}cm^{-1}$) x C x L (0.62 cm)

C = 0.006 / 9200 $m^{-1}cm^{-1}$ x 0.62cm

C = 0.006 / 5704

= 1.05 x 10^{-6} M/L

= 1.051 µmol/ L /min (convert to µmol x 1000 000)

= 0.00105 µmol / ml / min (convert to ml x 0.001)

5 µl in assay

therefore 0.00105 µmol / min / 5 µl

to convert to ml x 200

0.21 µmol / min / ml (TOTAL ACTIVITY)

To convert total activity to specific activity

Total activity x mg/ml protein in extract

9.13. AGES OF SEAHORSES IN THE TRIALS

Biofouling trial

Age at start of trial = 13 weeks old

Age at end of trial = 21 weeks old

Artemia enrichment trial

Age at start of trial = 7 weeks old

Age at end of trial = 15 weeks old

Ration trial

Age at start of trial = 7 weeks old

Age at end of trial = 15 weeks old

Copepod trial

Age at start of trial = 3 weeks old

Age at end of trial = 9 weeks old

Weaning trials

Age at start of trial = 13 weeks old

Age at end of trial = 21 weeks old